THE UNIVERSITY OF MANITOBA

LABORATORY TRANSMISSION OF WESTERN ENCEPHALOMYELITIS BY *CULEX TARSALIS* COQUILLETT AND ITS BEHAVIOR IN RICHARDSON'S GROUND SQUIRREL, *SPERMOPHILUS RICHARDSONII* (SABINE)

by

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ABSTRACT

Laboratory experiments were carried out on a Manitoba isolate of Western encephalomyelitis (WE) virus. A Manitoba strain of Culex tarsalis Coquillett was used for studies on transovarial and biological transmission of WE virus. The mosquitoes were infected by feeding upon viremic day-old chicks.

In the transovarial transmission experiments, females were allowed 2 additional blood-meals, each approximately 1 night following maximum egg laying. The offspring of each oviposition cycle was raised to the adult stage. Samples of each immature stage as well as the adults up to 3 weeks of age were assayed for virus. No WE virus was found in any of the F_1 generations.

It was found that female C. tarsalis more readily fed upon day-old chicks than mice. Both the chickens and mice were easily infected by the bite of a single infected mosquito. Transmission rates generally increased over 31 days to 100% by the end of the experiment. Infection rates remained at or near that level throughout the experiment. The Manitoba strain of C. tarsalis was found to be an efficient vector of the Manitoba isolate of WE virus.

The effect of a WE infection in the Richardson's ground squirrel was studied. Some infected squirrels were held at 24°C and some at 10°C. Hibernation appeared to arrest viral replication. Viremia persisted longer at 10°C than at 24°C. Recurring viremia was evident,

although of low titre. The squirrels were highly susceptible to WE inoculation, with a high rate of mortality (67%). Virus distribution in the tissues studied was not widespread, with the majority of isolations occurring in the brain. Histopathology revealed the basic brain lesions common to WE infection. Lesions were also found in other tissues, but because the animals were not raised under disease-free conditions, no definite conclusions could be made regarding the lesions. The squirrel appeared to be less than an optimum host of the virus.

Field studies on the squirrels indicated that they did become infected in the field. Although no virus was recovered from the blood samples of tested squirrels, 3 out of 93 sera collected in the summer of 1976 and in the spring and early summer of 1977 were positive for WE antibodies. The extent of the serologic survey was too small to predict widespread WE infection among these animals.

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CHAPTER I

LITERATURE REVIEW

History of Western Encephalomyelitis (WE)

In Canada, WE has been responsible for epidemics in both horses and man (31,48,52,53,67,68,133,146,199). Suspected cases of encephalomyelitis in horses were reported in Manitoba as early as 1932 (199), although Cameron (31) stated that equine cases have been periodically reported in the western United States and Canada since 1847. By 1935, the disease in horses was definitely recognized as WE in both Saskatchewan (67) and Manitoba (199). Until 1938, equine encephalomyelitis was considered primarily a disease of horses (51). The first major human WE epidemic in Manitoba took place during 1941 (52,148) when one of the largest encephalomyelitis epidemics in western North America occurred (133). Epidemics and related problems in Saskatchewan have been reviewed from 1935 to 1963 (49,50). More recent reviews of the arbovirus problem in Canada are given by McLean (148), McLintock (151), and McLintock and Iversen (156). Outbreaks of WE in humans in the United States up to 1963 are reviewed by Hess, Cherubin and LaMotte (109).

WE virus was first isolated from a brain of a horse by Meyer, Haring and Howitt (161) in 1930. The first laboratory transmission of the virus by an insect was performed by Kelser (124) in 1933 when he infected the mosquito Aedes aegypti (Linnaeus). WE virus was first isolated

from field-caught mosquitoes in 1941 (91). The species was Culex tarsalis Coquillett. Two years later, this species was confirmed as a vector when it experimentally transmitted WE to chickens (84).

Today, C. tarsalis is considered the primary vector of WE virus in western North America (33,37,38,54,89,92,110, 111,154,173,185). This conclusion is the result of years of study, both in the laboratory and in the field. C. tarsalis has been repeatedly found infected with WE virus in nature (148,154,173). It is the only mosquito species with the sufficient population numbers and incidence of virus-carrying mosquitoes to account for the occurrence of human and equine disease (18,57,154,210). Its seasonal activity coincides with the seasonal incidence of WE, and its feeding habits fit the known incidence of antibodies in vertebrate hosts (173). Together with these field observations C. tarsalis has proven itself an efficient vector of the virus in the laboratory (11,38,104,173,227).

Field Isolations of WE virus

WE virus has been isolated from many species of mosquitoes in North America, however the majority of isolations from any one species have come from C. tarsalis (33,64,120, 174). Table 1 shows the field isolations from mosquitoes that inhabit Canada. Other Aedes and Culex species (92,107, 128,216) not found in Canada have also yielded WE virus. The multitude of virus isolations from C. tarsalis compared

to those from other species can easily be seen in table 1. Besides mosquitoes, only the cone bug, Triatoma somguisuga (Le Conte), and 4 species of bird mites have been found naturally infected with WE virus (64). Most of the virus isolations from mosquitoes have occurred in the United States, indicating a need for more field work on virus-mosquito relationships in Canada.

Although C. tarsalis is considered the principal vector of WE in western North America (33,37,38,57,89,92, 110,111,154,173,185), the isolations of WE virus from other mosquitoes indicate that these species may also partake in the epidemiology of WE virus (174). The Aedes species may serve as amplifying vectors during the spring (154), or as possible assistants in the spread of WE among horses once an outbreak is underway (33). Culiseta inornata (Williston) may also be a significant transmitter of WE among horses in epidemics (154), or it may act as an overwintering host of the virus (110). Culiseta melanura (Coquillett) is considered the primary enzootic vector of WE in the eastern states (101). The virtual absence of WE in man and the apparent infrequency of WE infection in horses in this region is probably due to the fact that this mosquito species is an infrequent feeder on both these hosts (41). More work is still needed if definite conclusions are to be made on the importance of each species in the epidemiology of WE virus.

WE is not the only virus with which C. tarsalis has been found naturally infected. St. Louis encephalitis (SLE)

(28,90), California encephalitis (CE) (185), Turlock virus (28), and Flanders-Hart Park group virus (79) have been isolated from this mosquito species. These virus isolations increase the potential of C. tarsalis as an important disease vector in North America.

Laboratory Transmission of WE virus

There have been many attempts to transmit WE virus by arthropods in the laboratory (64). Transmission attempts by mosquito species of Canada are summarized in table 1. Those mosquitoes that have been found naturally infected with WE virus and can transmit it in the laboratory are potential vectors. These include Aedes dorsalis (Meigen), A. nigromaculis (Ludlow), A. sollicitans (Walker), A. trivittatus (Say), A. vexans (Meigen), Culiseta inornata and, of course, Culex tarsalis. A. fitchii (Felt and Young), although capable of transmitting WE virus experimentally, is in a unique position since no virus has been isolated from wild-caught specimens (104).

Although C. tarsalis has been studied primarily with regard to WE virus it has also been found capable of experimentally transmitting SLE (85), Eastern equine encephalitis (EE) (39), and Japanese B encephalitis (132). C. tarsalis is considered a principal vector of SLE in California (185).

Transovarial Transmission

One hypothesis for the maintenance of arboviruses in endemic areas is transovarial transmission by the vector.

Table 1. WE field isolations and laboratory transmission attempts with mosquito species found in Canada.

Species	Location of isolation and literature references	Transmission + or -, and literature references
Aedes campestris	Saskatchewan[154]	-[104]
A. canadensis	Massachusetts[101]	
A. dorsalis	California[92]	+[144,145]
	Colorado[229,43]	
	Saskatchewan[153,154,210]	-[104,108]
	Utah[206]	
A. flavescens	Saskatchewan[154,210]	-[104]
A. fitchii		+[104]
A. nigromaculis	Colorado[43]	+[145]
	Saskatchewan[152]	
A. sollicitans	Texas[169]	+[157]
A. spencerii	Saskatchewan[154]	-[104]
A. triseriatus	Iowa[192]	+[34]
A. vexans	Alberta[204]	+[125]
	Minnesota[25]	
	Saskatchewan[154]	
Anopheles earlei	Saskatchewan[152]	
An. freeborni	Washington[88]	-[84,108]
An. punctipennis	Iowa[192]	
An. quadrimaculatus		-[157]
Culex pipiens	Iowa[192]	-[157]
	Washington[88]	

Continued.....

Table 1. Continued

Species	Location of isolation and literature references	Transmission + or -, and literature references
C. restuans	Manitoba[168]	-[37]
C. tarsalis	Alberta[78,204]	
	California[60,82,182]	+[11,15,16,38,39,72,84,93,95,104,105,173,227,228]
	Colorado[43]	
	Iowa[243,192]	
	Manitoba[149]	
	Minnesota[25]	
	Montana[58]	
	Nebraska[58,92]	
	North Dakota[58,236]	
	Saskatchewan[153,154,210]	
	Texas[169]	
	Utah[59,205]	
	Washington[88,90,91]	
Culiseta inornata	Alberta[204]	+[84]
	Saskatchewan[153,154,210]	
	Washington[88]	-[104]
C. melanura	Alabama[216]	
	Georgia[41]	
	Louisiana[128]	
	Maryland[238,239]	
	Massachusetts[101]	

The adult female transmits the virus to her eggs while they are within the ovary (65,212). Laboratory studies on CE virus in Aedes species, as well as supportive field isolations from larvae suggest that CE virus can be maintained in nature by this mechanism (13,134,147,232,233,234). Furthermore, there is evidence that male A. triseriatus may transmit CE virus to the female while mating (230).

Further studies on transovarial transmission have shown that Venezuelan equine encephalitis (VEE) has been isolated from egg rafts laid by Mansonia perturbans (Walker) (36). C. quinquefasciatus Say has been reported to transmit SLE virus to its offspring. None of the F_1 adults were infected, however there was a low infection rate among the immature stages and most of the virus was found on the surface of the eggs (42).

Results on transovarial transmission of WE virus by A. aegypti (158) and C. tarsalis (11,38,86,227) have been negative. However, eggs laid by WE-infected A. triseriatus were positive for virus (129).

Vector Criteria

Guidelines for determining vectors of arboviruses have been established (7,8), and they are as follows: 1) isolation of the virus from the suspect vector in nature. The vector must be free from blood, which might contain the virus, 2) association of this arthropod with the vertebrate population in which the infection is occurring, 3) laboratory

infection of the arthropod with the virus by feeding upon an artificial substitute or a vertebrate host, and 4) laboratory transmission of the virus by the infected arthropod by its bite following a period of viral multiplication within the body of the vector (biological transmission).

Virus must be isolated from wild-caught mosquitoes to prove that infection in nature is possible, and it is important that these isolations occur when the arthropod is free of blood. Virus isolation from engorged mosquitoes may simply mean the virus was present in the blood-meal. This is no indication that the arthropod is capable of becoming infected.

For a vector-host combination to be important in nature, the vector and reservoir must be appropriately associated in both time and space (111,214). For example, C. tarsalis and birds are considered an important vector-host combination for WE maintenance (6,30,57,92,93,110,111,122, 127,153,155,174,185,188). The peak biting period of this mosquito species occurs at dusk, and the evening flights of birds to their roosting sites occur just prior to this time. Therefore, the birds are quiescent at the time of maximum feeding by C. tarsalis, and this would be a critical period for WE transmission (111).

Laboratory research on vector capability goes "hand-in-hand" with field studies. Three criteria for estimating vector potential in the laboratory have been established by Chamberlain, Sikes, Nelson and Sudia (37). They are the

infection threshold, infection rate, and transmission rate. The infection threshold is defined as the lowest concentration of virus capable of causing an infection in approximately 1 to 5% of the specimens of a particular mosquito species ingesting it. Other researchers have used a 50% level for infection threshold (11,104,227). The infection rate is defined as the percentage of mosquitoes in a feeding series that are found to contain virus, regardless of their ability to transmit. The transmission rate is the percentage of specimens of a given species transmitting infection by bite to susceptible animals after ingesting a meal having a high virus titre and after a suitable extrinsic incubation period. Reeves, Bellamy and Scrivani (183) formulated transmission and infection rates for mosquito vector populations in nature. A vector-potential gradient based on these 3 criteria would indicate relative mosquito vector efficiencies. The infection threshold and transmission rate are considered the 2 most important factors, with the former being more significant (37,40).

According to Mussgay (165) arbovirus establishment in an arthropod depends upon 3 factors. These are 1) virus-arthropod specificity (the susceptibility of the arthropod to the virus), 2) the manner and route of infection, and 3) the infection threshold. These factors are interrelated. The infection threshold not only depends upon the manner and route of inoculation but also upon the arthropod species (40,165). This threshold phenomenon is considered as a

"gut barrier" to infection (40). If a high enough concentration of virus is ingested to overcome the "barrier", infection of the mosquito will result. The rest of the tissues are readily infected.

Other characteristics of vector populations are important in determining success of pathogen transmission (8,37, 96,111,129,175,177,185). These include: numerical abundance, the extent of its innate susceptibility and ability to transmit, longevity at temperatures that favour completion of extrinsic incubation of the virus, the presence and extent of autogeny since high autogeny rates reduce WE transmission, affinity of the vector for a vertebrate species that can circulate the pathogen in its blood in an infective dose for the vector, the dispersal or flight range of the vector, and the extent of repeated blood-feeding by the vector. Reeves and Hammon (185) believe that if other factors remain constant, there is a critical vector population below which virus will not be transmitted. If the vector population exceeds this threshold, transmission accelerates. Reeves (180) amplifies this hypothesis by stating that below a specific population level the rate of feeding by the vector on, and virus transmission to, the aberrant hosts are so low that the chances of a clinical infection developing are practically zero. Nevertheless there could still be a low incidence of latent infections in man.

Climatic Conditions Affecting Vector Competence

The environment of a mosquito species dictates the degree to which an arbovirus will be transmitted. Not only does it affect the vector, but it may also have an influence on the virus itself. Climatic factors can be broken down into 2 main components: water supply and temperature.

The amount of water available to the breeding vector population determines its abundance. Culex and Culiseta species, being multivoltine, depend indirectly on rainfall because they lay their eggs on permanent types of standing water. The increasing use of irrigation in agriculture provides more suitable water for the build-up of mosquito populations, particularly C. tarsalis (148). Aedes species depend mainly on rainfall of the current season because many are univoltine and lay their eggs in temporary pools (150,153). Spring runoff influences vector populations. In 1952, Kern County, California, experienced its worst spring flood in a 10 year study. That year, the area encountered peak vector populations and a WE epidemic (185). The principal ecological indicators of WE epidemics in California are rain, snow surplus and flooding (96). The relative humidity of the mosquito's microclimate may also play a role, as it affects its longevity (111).

Temperature directly affects the extrinsic incubation of an arbovirus in its vector as well as influencing vector longevity and abundance (111,150,153,165,177, 185). Since mosquitoes are poikilothermic there is an

inverse relationship between temperature and the extrinsic incubation period of the virus (165). Increasing the temperature generally decreases extrinsic incubation of the virus. In Saskatchewan, the majority of WE isolations occurred during a period when mean weekly temperatures ranged between 18°C and 24°C (153). Therefore, the effective temperature for incubation of the virus in Saskatchewan mosquitoes was within this temperature range.

Temperature affects the relative abundance of mosquito species. The abundance of C. tarsalis increases during high temperature while that of C. inornata decreases (150, 153). Culiseta is more abundant at lower temperatures. For the univoltine Aedes, temperature only serves to retard or hasten their development.

Winter weather also affects the epidemiology of WE virus (153). Hosts such as mosquitoes, garter snakes, frogs and ground squirrels, are forced to hibernate or, as in the case of many wild birds, migrate south. WE outbreaks have followed severe, cold winters. The fall and in turn the early spring weather determines the exact time the hibernators go into and come out of hibernation.

Hardy and Reeves (96) conclude that mosquito populations are made up of virus susceptible and virus resistant individuals that can be genetically determined. Therefore, certain environmental factors, such as temperature, can select for the virus susceptible or resistant component in nature. Andrewes (5) states that normally arthropod-borne

viruses exist in a state of symptomless equilibrium with their natural host. When the ecology of the vectors is favoured by certain climatic changes, the virus spreads wider among its normal hosts, and then a spillover to abnormal ones occurs. A good outline of the factors that influence the probability of encephalomyelitis epidemics is given by Reeves (178) in which he goes over the variables, and the methods for measuring them.

WE Hosts

The basic transmission cycle generally accepted for WE virus is bird-mosquito-bird, with C. tarsalis the endemic and epidemic vector, and wild birds, especially nestling birds, the primary reservoirs (6,30,57,92,93,110,111,122, 127,153,155,185,188). Domestic fowl are effective hosts, but are not essential to WE maintenance (127,185). There is some evidence that small mammals may be important as spring amplifying hosts (119,126,137,245). Most researchers believe that mammals, such as horses and man, are incidental hosts of WE - victims of an overflow of virus from its basic bird-mosquito-bird cycle (23,93,110,153,200). However, some researchers believe that mammals as well as birds act as reservoirs for infecting mosquitoes (51,83,122,123,139,176). Eklund (57) believes that the evidence indicating mammals as maintaining WE virus is inconclusive.

If an animal species is going to be important in the transmission cycle of WE, it must have the following

characteristics (93,127,185): it should 1) be abundant, 2) show no apparent signs of infection, 3) have as a result of a small peripheral inoculation a reasonably large amount of virus circulating in its blood for more than a fleeting period of time, 4) not bestow a first season's protection to its offspring by maternal transmission of antibodies, and 5) be a preferred host of the mosquito vector. Birds fit 4 of these criteria well, but they transfer maternal antibodies to their offspring (115,200). However, the young soon lose this immunity (122).

WE virus has been isolated from a wide range of animals (6,23,30,43,62,98,107,114,120,131,141,153,209,215, 239). Twenty species of birds and 6 species of mammals have yielded WE virus (111). Reptiles and amphibians have also been found infected (29,70). The first isolation of WE virus from a host other than man or horses during an epidemic occurred in North Dakota in 1941 (47). Cox, Jellison and Hughes considered it important that this isolation was from a bird, rather than a mammal.

Many birds, mammals, amphibians, and reptiles have undergone laboratory inoculation to test their susceptibility to WE virus (29,73,94,115,129,130,159,163,211,219,221,240, 235). Burton, Connell, Rempel and Gollop (26) discovered that wild ducks could be infected orally. The cat, dog, sheep, swine, goat, fox, ferret, rabbit, and opossum were found refractory to WE virus (73,163,221). Recent research indicates that sheep and cats are resistant (32). No avian

species has been demonstrated to be refractory to infection with WE virus (214).

Serologic surveys for WE antibody have been extensive, covering most western states and provinces (3,23,26,27,30, 43,81,82,92,98,107,113,116,117,128,171,185,190,208,215,237, 239,243). Although they indicate which individuals are exposed to the vector and which are capable of becoming infected with WE virus, they do not reveal when the virus was there, what it did nor which hosts are capable of infecting more vectors (126,127). WE antibodies have been found in more than 75 species of wild birds and a half dozen species of wild mammals, as well as most of the common domestic birds and mammals (111). Reptiles and amphibians have also been found with WE antibodies (29,211). Buffalo and reindeer in northern Saskatchewan have yielded neutralizing antibodies to WE (27). This is outside the known geographic range of C. tarsalis.

The Richardson's ground squirrels (RGS) have been implicated as maintenance and/or amplifying hosts of WE virus (137). WE virus has been isolated from wild-caught RGS (30,76,139). The early spring isolation of the virus from a squirrel by Burton, Spalatin and Rempel (30) indicated a possible latent infection because there was no mosquito activity before that time. However, research done by Leung (138) indicates the unlikelihood of a latent infection in RGS.

Serologic surveys on WE antibody in RGS in Saskatchewan indicate high infection rates in the epidemic year of

1965 and low infection rates during non-epidemic years (139). The maximum number of seropositive squirrels along with virus isolations occurred during June, the month of peak squirrel abundance and activity. Experimental work indicates that mosquitoes are capable of becoming infected from feeding upon a viremic squirrel (137). These factors provide support for the possibility that the squirrels are early season amplifying hosts of WE virus.

Experimental inoculation indicates that RGS is very susceptible to WE infection (75,115,137,219,220). Mortality rates depend upon the route of infection, with more deaths following intranasal or intracerebral inoculations than following subcutaneous inoculation. The pathogenesis of the virus in RGS is related by Leung (137). The pathogenesis of WE infections in mice has been studied more intensely (1,2,66,140,197). The effects of WE infections have also been studied in guinea pigs, the mongolian gerbil, and horses (104,118,159,161,197). A general description of the effects of virus diseases in mammals is given by Bang and Luttrell (9), Smith, Jones and Hunt (207), Mims (162), and Fenner and White (63).

Host Preference of *Culex tarsalis*

C. tarsalis feeds on a wide range of animals, notably avian and mammalian hosts (12,55,74,106). Not only is this characteristic an indication of its broad adaptation, it is also important in the transmission of encephalitis viruses

(10,129,224). C. tarsalis feeds sufficiently upon horses and man to propagate an epidemic at times when its population is high. Although C. tarsalis feeds on a wide range of animals, it prefers avian hosts to other vertebrates (12, 55,120,167,189,196,203). In areas where the mosquito has equal opportunity to feed on either mammals or birds, the latter are more often selected (4).

Of its avian hosts, C. tarsalis generally prefers chickens, passeriform, columbiform and strigiform birds (4, 103,184,196,224,225). Cattle are the most preferred mammals (184,196,224,225). Horses, dogs and cats are other favoured domestic blood sources (4,225), while rabbits are the most frequently contacted wild hosts. Other wild mammals, such as rodents, do not contribute significant numbers of blood-meals (191,225). Humans are not common hosts (120,184). Blood-meals are seldom taken from reptiles or amphibians (191,225,226).

C. tarsalis demonstrates a seasonal shift in its feeding pattern, from a predominance of feeding on birds in the spring and early summer to feeding on significant numbers of mammals in mid- and late summer (4,74,103,224,225). Mammalian feedings peak in August and September. This increase in mammalian feedings coincides with the appearance of WE virus activity in mammals and man (223).

The seasonal shift in host preferences may be due to the coincidental increase in C. tarsalis populations (103, 167). Reeves (180) believes this divergence results from

most bird species being intolerant to attack by large numbers of mosquitoes. Other reasons postulated for this host preference shift are: 1) Some seasonal physiologic change in C. tarsalis that affects its host preferences, 2) a preference for young mammals that are more abundant in the summer, 3) an undetected increase in cattle and rabbit populations in the mid-summer period, 4) a behavioral change in the mammalian host, 5) changes in roosting sites which lead to decreased accessibility to birds, 6) qualitative changes in host population, ie the number of young or nestling birds present, or 7) the birds become less attractive as they mature (180,225).

C. tarsalis prefers to feed on nestling or immature birds rather than adult birds (20,55). Young birds are nearly free of feathers and they are more quiescent than adults. These factors may be the reasons for this preference. When young and adult birds are placed together in cages, a greater percentage of the attracted mosquitoes feed on the younger birds (20). Since the nestling birds rather than the adults are more frequently used as hosts by C. tarsalis, they may be more important as reservoirs of encephalitis viruses. Consequently, birds that nest during the encephalitis season are more involved in virus maintenance than those that do not.

Detection of multiple feedings by C. tarsalis has been limited. This may be due to limitations of the precipitin test, rather than C. tarsalis confining its feeding

to a single host (56,203). All combinations of feeding on multiple hosts cannot be detected. Multiple feedings on hosts of the same species or on the same host cannot be assessed by the precipitin test. Also, the test cannot detect multiple feedings with long periods of time between them. Therefore, precipitin data represent the minimum number of multiple feedings which have occurred. Birds provide most of the multiple feedings, followed by cattle, sheep, dogs, and horses. Humans, hogs, and rodents provide small percentages in these multiple-feeding patterns (56). Multiple-feedings by a vector are essential for the spread of virus.

Winter Maintenance of WE Virus

Several hypotheses have been postulated for the persistence of WE virus in certain endemic areas (7,111,175, 181). The most common of these are: 1) Migrating birds carry the disease northward with them in the spring, 2) the virus survives the winter as chronic infections in various vertebrate hosts, but probably avian, 3) other arthropods besides mosquitoes harbor the virus during the winter and infect susceptible birds during the arthropod's most active stage, 4) infected mosquitoes hibernate through the winter and infect susceptible hosts the following spring, and 5) the virus survives the winter by transovarial transmission in its mosquito vector.

Circumstantial evidence (128,141,188) indicates that

arboviruses can be transported by migratory birds. The early spring isolation of WE virus from a migrant bird (morning dove), before mosquito transmission is likely, supports this hypothesis (30). The migratory blackbird has been suggested as an important host of WE virus in California (122). In contrast, results obtained by Kissling, Stamm, Chamberlain and Sudia (130) weaken the assumption. In their study, no virus was isolated from birds wintering in the southern United States nor from those entering the United States on their northward spring migration. Eklund (57) and Johnson (123) both believe that there is no evidence that the virus is introduced into the northern United States and Canada by migratory birds on their northward spring migration. The southward transportation of WE virus by migratory birds may be more probable (122). It is generally believed that resident avian hosts or arthropod vectors are more likely to be winter reservoirs (111).

Vertebrate hosts considered important as overwintering reservoirs are snakes, birds and rodents. Experimental evidence indicates that overwintering of WE virus is quite feasible in snakes (29,69,70,71,211). Snakes experience a cyclic viremia, pass the disease to offspring and can carry it through hibernation if they hibernate at the right time. Winter carryover of WE virus by birds is possible since latent WE infections have been recorded in birds (189). Rodents, especially ground squirrels, have been suggested as overwintering hosts (139,220). Hibernating Richardson's

ground squirrels are unlikely reservoirs since WE virus infections interrupt hibernation (138). Early WE virus isolations from a mouse (155) and early seasonal transmission of WE virus in snowshoe hares (119,245) before the commencement of mosquito activity indicate a possible mammalian reservoir. Isolations of WE virus from potential winter reservoirs can either be due to persistent infection or continuous nonarthropod transmission (123).

Both mites and ticks have been implicated in the overwintering of WE virus. Although WE virus has been isolated from mites of wild birds (186,187,218) and chickens (217), laboratory investigations indicate that they do not play a significant role in WE maintenance (35,241). Some ticks are capable of passing WE virus to their offspring transovarially, and the larval, nymphal, and adult stages are capable of infecting susceptible hosts (222). Other tick species are not capable of becoming infected, or if they do, they cannot transmit the virus (97). Field isolations of virus from ticks as well as more laboratory studies are necessary to confirm their role in the epidemiology of WE virus.

Reeves (175) believes that mosquito-borne viruses may have an adult female mosquito reservoir, however there is little evidence to support this suggestion. A host of researchers have shown that overwintering of WE virus in C. tarsalis is unlikely because: a) Winter and spring isolations of WE virus are too few (22,88,92,182,193,194,

195,196,244). b) A high majority of the overwintering females are unfed since blood-feeding decreases in the fall (14,17,21,24,54,166,182,193), and blood-fed individuals have a lesser chance of surviving the winter (16,88). Shemanchuk and Morgante (204) concluded that they had found infected C. tarsalis entering hibernation in mammalian burrows in August. However, such sites are common resting habitats for these mosquitoes during the summer (100,154). c) Although WE carryover in C. tarsalis has been demonstrated experimentally in California, it is doubtful that it occurs in nature (15). There were also signs of WE attenuation in these mosquitoes after 8 months. C. inornata has more potential as an overwintering reservoir than C. tarsalis. A higher percentage of this species hibernate after taking blood-meals (54,204).

It is unlikely that transovarial transmission of WE virus occurs in C. tarsalis (11,38,86,227). This overwintering mechanism would be of little importance since most C. tarsalis hibernate as unfed adult females (111). Aedes species may be capable of carrying the virus through the winter in this manner.

Evidence for the mechanism of WE virus maintenance during the winter is not conclusive (110). More research is needed before definite conclusions can be made.

This review of literature clearly indicates that a vast amount of research has already been completed on the epidemiology of WE virus and its principal vector C. tarsalis.

The basic disease transmission cycle has been discovered, including the principal reservoirs. Attempts have been made to discover the overwintering mechanism, and factors leading to an epidemic. However, more research is still needed, into such areas as the full range of WE distribution, its method of dissemination, its persistent long term reservoir and its complete range of vector species (8,176).

CHAPTER II

LACK OF TRANSOVARIAL TRANSMISSION OF WESTERN ENCEPHALOMYELITIS BY *CULEX TARSALIS*

INTRODUCTION

Transovarial transmission has been suggested as a mechanism for maintenance of viruses within endemic areas. This mechanism could be important in temperate zones where vector activity is periodically reduced by adverse climatic conditions. The disease agent must be carried through each sequential developmental stage to the adult, and be present in sufficient concentration to be transmitted by bite to susceptible hosts. If this occurred, then transovarial transmission could be a significant factor in virus maintenance.

Culex tarsalis is considered the principal epidemic vector of Western Encephalomyelitis (WE) in many parts of North America. The importance of *C. tarsalis* in the epidemiology of WE would be amplified if it was capable of transovarially transmitting the virus. This could explain how the virus is maintained throughout the year in temperate zones.

The purpose of the following study was to determine whether a Manitoba strain of *C. tarsalis* could transmit a local strain of WE to its offspring.

MATERIALS AND METHODS

VIRUS

The WE strain used in the 3 experiments was originally isolated from a horse brain during the Manitoba epidemic of 1975. It has undergone 3 successive passages in Vero cell tissue culture. Identity of the virus was confirmed by the National Arbovirus Reference Centre. The titre of the stock virus was log 5.3 $TCID_{50}$.

CELL CULTURE

Virus detection in mosquitoes and titrations of donor chick blood samples were done on Vero cells (green monkey kidney) which were originally obtained from the American Type Culture Collection. The growth medium was #1969 containing 10% bovine serum, 1% L-glutamine and antibiotics: 20,000 ug of penicillin, 10,000 ug of streptomycin, 0.50 ug of neomycin (lxanti) and 5,000 iu of mycostatin per 100 ml. It was buffered with 8% sodium bicarbonate and 10% hepes solution.

The cells were routinely transferred by trypsinization with a 0.25% trypsin solution. Each plastic flask yielded a maximum of 50 tubes of Vero cells, each with 2 ml. of media-cell suspension, for virus assay the following day. Infected cells were maintained in a medium equivalent to

the growth medium, but with 1% bovine serum and no L-glutamine.

CHICKS

The day-old chicks used for infecting the female mosquitoes were obtained from a local hatchery. They were inoculated intramuscularly with 1000x$TCID_{50}$, 18 hours before the mosquitoes were allowed to feed on them. One chick was sacrificed at the commencement of the feeding period, and the rest were killed at the end, a total of 9 hours apart. The chick blood was diluted 1:4 in the anticoagulant Alsevers with 2 x antibiotics. These samples were centrifuged at 4000 rpm, and then titred on Vero cells.

MOSQUITOES

The C. tarsalis used in the experiments were offspring from a permanent Manitoba colony kept by the Entomology Dept., at the University of Manitoba. All larvae were reared under a photoperiod of L:D 16:8. The adults were maintained at the same photoperiod and at 75% relative humidity.

All female mosquitoes that had fed upon the infected chicks were removed to other cages. Oviposition dishes were provided 4 days after each blood-meal, and left there for a period of 48 hours. The female mosquitoes were offered two additional non-infective blood-meals following the first infective one.

The egg rafts were collected after each oviposition cycle, and the larvae were reared to different developmental stages before virus assay. In the first experiment, all larvae were reared to the adult stage, and were one week old when frozen at -70°C. During the second and third experiments all instars of larvae and the pupae were sampled, and tested for virus. Finally, adults that ranged in age from 1 week to 3 weeks, were tested for virus.

VIRAL ASSAY

The method for preparing mosquitoes for virus assay was adopted from that described by Stackiw (213). All mosquitoes were crushed in bijou bottles containing small plastic beads and diluent, using an electric stirrer. The diluent was MEM containing 20% bovine serum and twice the regular dosage of antibiotics.

The infected females were ground in 1 ml. of diluent after the third ovarian cycle. All offspring were ground in 2 ml. of diluent after pooling (not exceeding 54 individuals). The adults were sexed before pooling. The pooled extracts were centrifuged at 4000 rpm for 20 minutes before the supernatent was inoculated onto tissue culture.

Blood samples from chicks were titred by inoculating 10-fold dilutions onto Vero cell tubes.

All inocula were allowed to absorb for at least 1/2 hour at room temperature. Then the cells were rinsed with Hanks medium containing 2xanti, and maintenance medium was

finally added. The inoculated monolayers were observed for 6-7 days for cytopathic effects (figures 1-4). The $TCID_{50}$ endpoints were determined by the Kärber method (136). The identity of virus recovered from the host blood samples and the mosquitoes was confirmed by the neutralization test (136) with chick antisera on Vero cells.

RESULTS

In the first 2 experiments, all the parent female mosquitoes surviving the third ovarian cycle were positive for virus. Only 50% of those in experiment 3 contained virus. This low percentage positive was probably due to low viremia in the donor chicks which ranged from 0 to $\log_{10}$ 5.2 $TCID_{50}$. Viremias in the other donor chicks were log 5.1-7.1 $TCiD_{50}$ for experiment 1, and log 6.2-7.5 $TCID_{50}$ for experiment 2.

There was no evidence of transovarial transmission in any of the 3 experiments. All pools of larvae, pupae and adults were negative (table 1). In experiment 1, 26 adult pools, comprising 603 males and 607 females were tested. In experiment 2, 333 larvae, 224 pupae, 328 males and 299 females were tested in 13 larval pools, 9 pupal pools and 220 adult pools. In experiment 3, 271 larvae in 12 pools, 190 pupae in 8 pools and 220 males and 209 females in 22 pools were tested.

Figure 1. A confluent monolayer of Vero cells (x11.6).

Figure 2. A monolayer of Vero cells showing cytopathic effect (CPE) (x11.6).

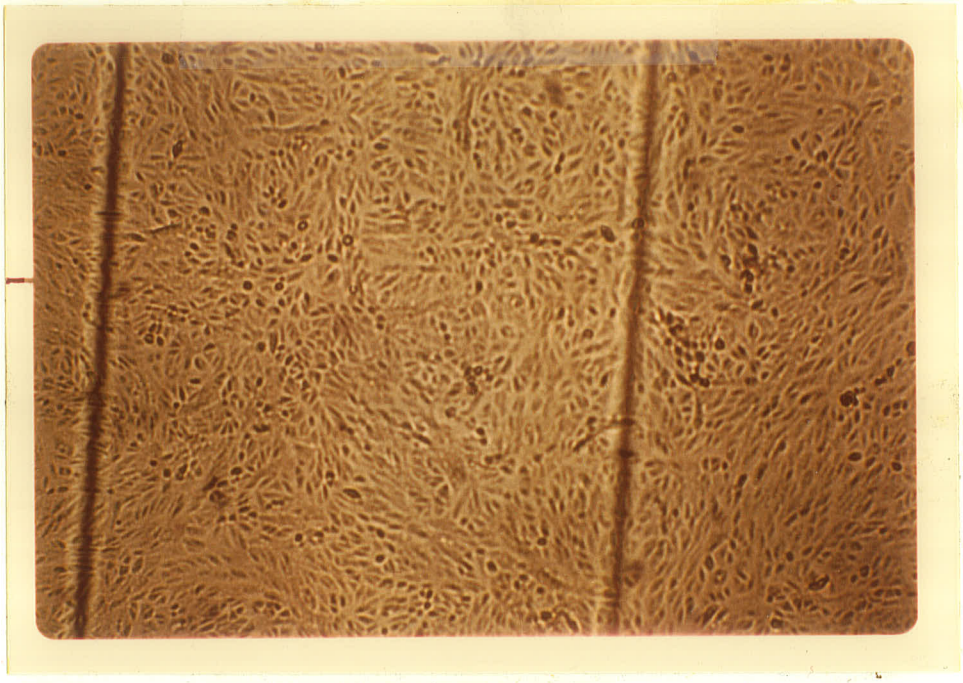


Fig. 1.

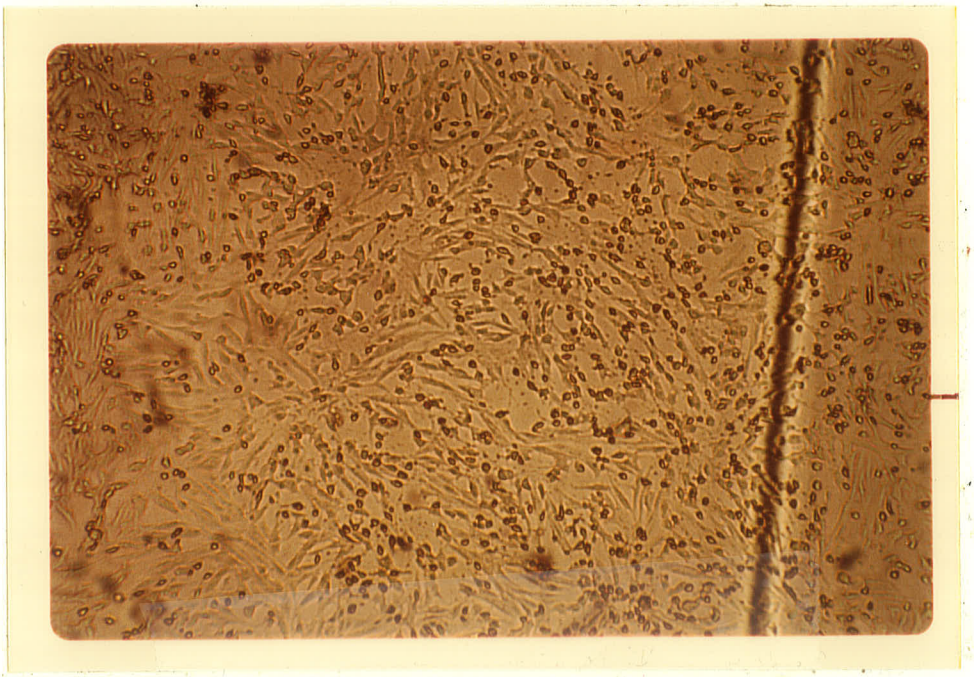


Fig. 2.

Figure 3. A more advanced state of CPE in Vero cells (x11.6).

Figure 4. A final state of CPE in Vero cells; the cells are completely "rounded-up" (x11.6).

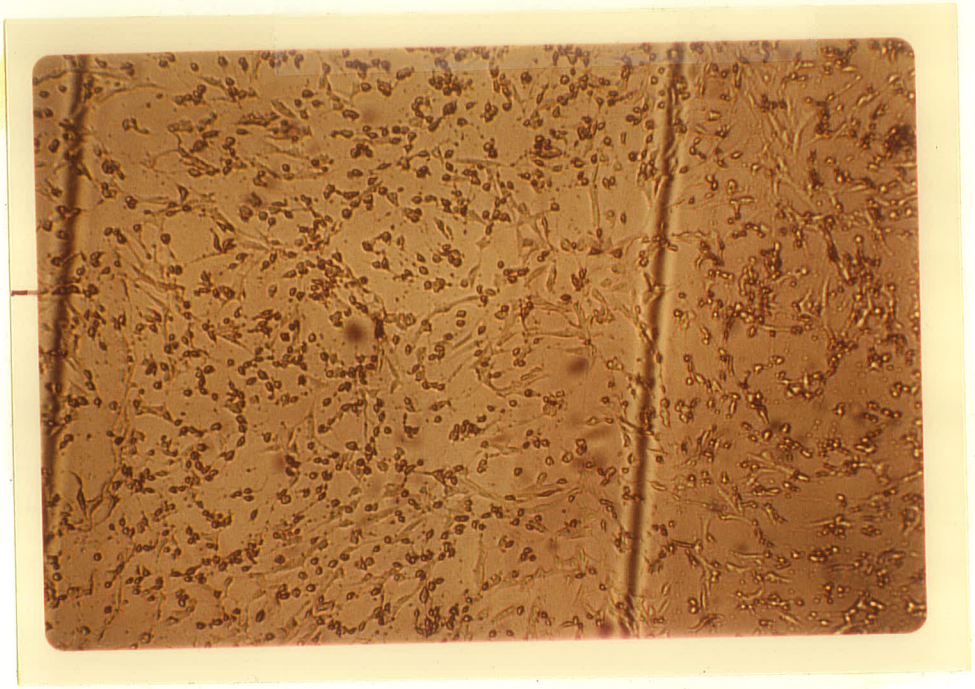


Fig. 3.

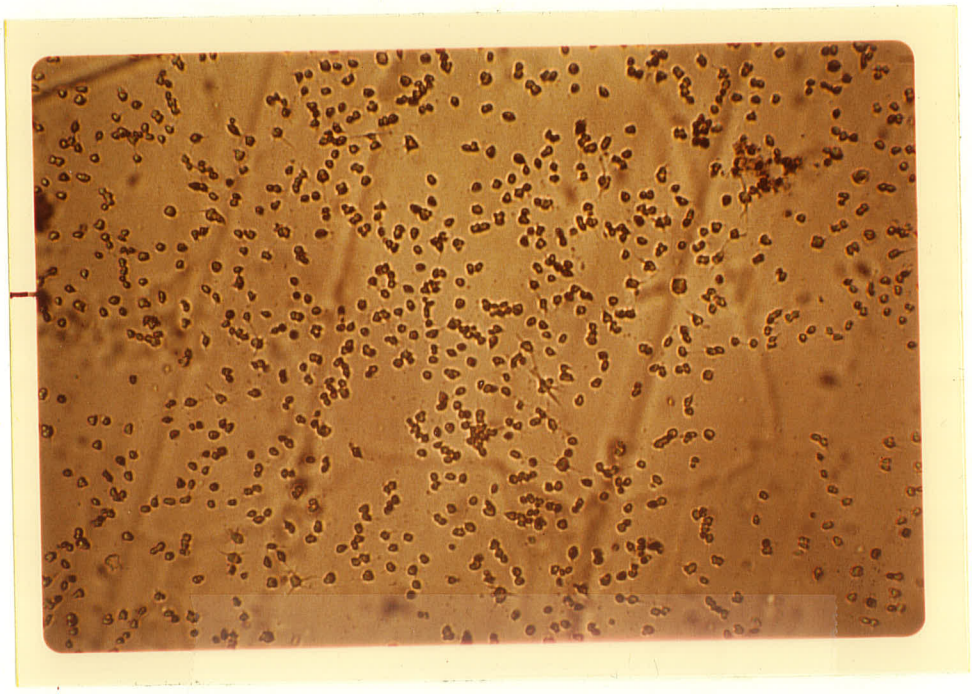


Fig. 4.

Table 1. Number of mosquito pools tested. Each pool represents different samples of the progeny of 3 ovarian cycles from infected Culex tarsalis.

	Exp. 1.			Exp. 2.			Exp. 3.		
Ovarian cycles / Developmental stage	1	2	3	1	2	3	1	2	3
Larvae				3	6	4	3	6	3
Pupae				6	1	2	2	5	1
Adults									
1 wk old	5	13	8	5	3	2	4	2	2
2 wk old				3	2	2	4	2	4
3 wk old				3	2		2	2	

DISCUSSION

The lack of virus in the F_1 generations of infected females in these experiments agrees with earlier research on WE with C. tarsalis (11,38,86,185,227). Progeny of infected adults have been tested for virus and found negative. However, it should be noted that Thomas (227) found 10% of the egg rafts from infected adults were contaminated with virus. No adults were raised from these infected egg rafts. Other experiments on egg rafts of infected adults yielded negative results (38,86).

WE has not been detected in the ovaries of female C. tarsalis until the fourth day after the infective bloodmeal (227). Virus concentration reached a maximum 10 days after infection. Therefore, one would be more likely to

find virus in progeny from ovarian cycles at this time. Such is the case with C. quinquefasciatus infected with SLE (42). No virus had been detected on eggs laid within 8 days following vector infection, however, 92% of the eggs laid after this time were contaminated with virus. Most of the virus was found to be on the outside of the eggs. This could be what Thomas (227) found in his experiments with C. tarsalis and WE virus. Since no virus has been found in the immature or adult offspring of infected C. tarsalis, surface egg contamination seems more likely than penetration of the eggs by the WE virus.

There is still the possibility that hatched first instars could become infected by feeding on the surface of virus-contaminated egg rafts. In the laboratory, mosquito larvae have been infected by placing them in media containing virus (44,45,112,170). Adult females raised from these larvae were able to transmit the virus with their bite. This may also explain the isolation of some viruses from field-caught larvae (134,147,234). No WE virus has been isolated from wild-caught male C. tarsalis, although it is known that they can harbour the virus for at least 1 week (185). Similarly, no virus has been isolated from female adults raised from field-caught larvae (87).

Perhaps the reason C. tarsalis is unable to transmit WE transovarially is related to the structure of its ovaries (19). There are two sheaths, interposed between the body cavity and the basement membrane of the egg

follicles, that may serve as a barrier to virus entry. Ticks, in comparison, lack these 2 sheaths and are able to transmit some alpha viruses. The tick, Dermacentor andersoni Stiles is capable of transmitting WE to its offspring (222).

Further evidence against transovarial passage of WE by C. tarsalis lies in the mosquito's ecology. Culex mosquitoes overwinter as adults, in contrast to Aedes which overwinter as eggs. The latter is known to transmit La Crosse virus, a CE group virus, (13,233), transovarially. Watts and Eldridge (231) suggest that the latter form of overwintering favours the selection of transovarial transmission, while the former method does not. Transovarial passage may be of little or no importance for virus maintenance in mosquitoes that overwinter as adults.

CONCLUSION

C. tarsalis appears incapable of transmitting WE virus to its F_1 progeny. This may be due to the structure of its ovary, or its ecology. Since C. tarsalis overwinters as an impregnated female adult, this mechanism may not be important for virus maintenance. The capture of male C. tarsalis in the field and the laboratory rearing of field-caught larvae with no isolation of WE virus, support this negative conclusion.

CHAPTER III

BIOLOGICAL TRANSMISSION OF WESTERN ENCEPHALOMYELITIS VIRUS BY *CULEX TARSALIS*

INTRODUCTION

C. tarsalis has been confirmed as the principal epidemic vector of WE in western North America (33,37,38,111, 154,156,173). The virus has been isolated from this mosquito species in many states south of the Canadian border - California (185), Washington (88,91), Utah (59,205), North Dakota (120), Iowa (192,243), Nebraska (92), and Minnesota (25). In Canada, WE has been obtained from field-caught *C. tarsalis* in Saskatchewan (153,154,210), Alberta (78,204) and Manitoba (149).

In Manitoba, WE virus has also been isolated from *C. restuans* Theobald (168). In 1966, the virus was also recovered from a pool of *Culex* species at the Delta Research Station, on the south shore of Lake Manitoba (143). No further attempts were made until 1975, when another WE epidemic occurred. WE virus was isolated from field-caught mosquitoes that summer, but the infected mosquitoes were not separated to species (201).

Equally important as field studies in determining vector competence are laboratory studies on the mosquito's ability to become infected with virus by feeding upon a viremic host and transmitting the virus by its bite to another host. Important considerations in determining vector ability are infection threshold, infection rates, and

transmission rates (previously defined, pg. 9). Observations on these 3 factors were carried out with Manitoba strains of C. tarsalis and WE virus.

MATERIALS AND METHODS

VIRUS

The stock virus used in the following experiments was the same as that used previously (Ch. 2).

CHICKS

Day-old chicks were obtained from a local hatchery. Those used as donor chicks in the experiments on infection and transmission rates were inoculated intramuscularly (i.m.) with 0.03 ml. of virus, dosage 1000 x $TCID_{50}$, 15-18 hours before exposure to mosquitoes. Viremias were determined by taking blood samples from the chicks prior to and at the end of mosquito feedings. The length of the feeding period ranged between 15 and 27 hours after i.m. inoculation of the chicks.

The chicks used as secondary hosts were strapped to 1x6" cages, each containing 1 mosquito. These chicks were left exposed to the mosquitoes for 9 hours. All chicks which had been fed upon by mosquitoes were observed for 5 days. If death or CNS symptoms occurred within this 5 day period, the brains were removed for virus assay. Chicks showing no signs of infection within 5 days were considered to be negative.

The chicks used in the experiment on infection threshold were also inoculated with 0.03 ml. of 1000 x $TCID_{50}$ of virus. Mosquito feeding commenced 3 1/2 hours after chick infection and continued for 5 hours. At 1 hour intervals, the viremic chicks were strapped to cages containing mosquitoes and left there for 1/2 hour. Immediately after each 1/2 hour feeding, the chick was killed and exsanguinated, yielding a volume of blood no greater than 1 ml. The blood samples were used to determine the level of virus available to the mosquitoes.

To prevent coagulation, all chick blood samples were added to 2.5 ml. of Alsevers solution containing 2 x antibiotics. The blood samples were frozen at -70°C before being thawed and centrifuged at 4000 rpm for 20 minutes. The supernatant was used for virus titration.

MICE

The mice, used as secondary hosts in the transmission experiments, were obtained from disease-free colonies held at either the Agriculture Services Complex or the Animal Science Building, University of Manitoba. Those mice which were fed upon by mosquitoes were kept for 10 days in isolation. If death occurred within this 10 day period, virus re-isolation from the brain was attempted to confirm infection. Otherwise, serology using the HAI test (described later, pg. 38) was performed on the sera of surviving mice to detect antibody response from infection. Blood samples

were centrifuged at 2000 rpm for 10 minutes, and the serum removed and frozen for HAI testing.

MOSQUITOES

The mosquitoes used in these experiments were from the same stock previously mentioned (Ch. 2). All experiments were conducted at 24°C. The mosquitoes were deprived of a sugar source at least 48 hours before they were allowed to feed upon infected chicks. Feeding was determined visually and all engorged females from each infection attempt were placed in separate cages, forming experimental groups. Oviposition dishes were supplied to these mosquitoes 4 and 5 days after the infective blood-meal. Mosquitoes were periodically removed from these groups for infectivity tests and/or transmission attempts to chicks or mice. An interval of at least 4 days elapsed before mosquitoes were allowed a second blood-meal.

In the experiment on transmission rate, mosquitoes were separately placed in 1x6" cages for individual feeding on secondary hosts. Mosquitoes that fed for the second time were harvested and stored for virus assay.

In the infection threshold experiment, 5 groups of 30 mosquitoes were aspirated into small 1x6" cages. All mosquitoes that fed during the 1/2 hour that the chicks were strapped to the cages were kept for 12 days before preparation for virus assay.

TISSUE CULTURE

Vero cell tissue culture was used for virus assay of chick blood, host brain tissue and mosquitoes. Its maintenance and growth have previously been explained (Ch. 2).

VIRUS ASSAY

Each mosquito was ground in a bijou bottle with 0.4 mm. glass beads on an electric mixer in 1 ml. of MEM containing 2 x antibiotics. Chick and mouse brains were crushed in 2 ml. of diluent. These were centrifuged at 4000 rpm for 20 minutes before 0.4 ml. of the supernatant was inoculated onto 2 tissue culture tubes. For donor chick blood titres, the blood was inoculated in 10-fold dilutions onto the Vero cell cultures. CPE (cytopathic effects) first showed within 48 hours.

SEROLOGY

The haemagglutination inhibition (HAI) test used to determine antibody titre in mice having no apparent WE infection, was modified from Sekla and Stackiw (201). The basic method was originally described by Conrath (46).

The haemagglutinating antigen, in inactivated form, was obtained from the National Arbovirus Reference Laboratory at the University of Toronto. The diluent for the entire test system was an organic buffer (HSAG) containing 0.596% N-2 hydroxyethyl piperazine N-2 ethane sulfonic acid (HEPES), 0.1% normal human serum albumin and 0.00025% gelatin,

prepared by Connaught Medical Research Laboratories. Mouse serum was absorbed with packed day-old chick RBC after treatment and prior to the HAI test. All reagents were used at half the volume of those used by Sekla and Stackiw (201).

For the removal of nonspecific HAI inhibitor in the mouse serum, 0.3 ml. of 1:3 diluted serum samples were each incubated with 0.1 ml. of sodium heparin-manganous chloride solution (5000 units of heparin and 1 molar $MnCl_2$/ml.), at $4^{o}C$. for 30 minutes. Then 0.4 ml. of HSAG was added to make a 1:8 dilution of serum. The precipitated inhibitor was removed by centrifugation at 2000 rpm at $4^{o}C$ for 20 minutes. Treated serum was recovered with a pipette.

The treated serum was absorbed with packed chick RBC to get rid of any anti-RBC factors which may have been present. Initially these chick RBC were washed at least 3 times with phosphate buffered saline. They were finally washed with dextrosgelatin vernal buffer before serum absorption. Absorption was allowed to proceed at room temperature for 1 hour, then the serum was centrifuged for 15 minutes at 2000 rpm, and the absorbed serum recovered.

Prior to the HAI test, the antigen was titred to determine at what dilution it should be used in the test. Antigen was serially diluted in 10 wells with HSAG, then 0.025 ml. of a 0.4% suspension of RBC was added. This was mixed well and left for 1 hour at room temperature. The last well at which the antigen haemagglutinated the RBC was taken as 1 HA unit. Since 4 HA units were necessary,

2 wells toward the stronger antigen concentration was used as the antigen dilution.

In the HAI test, serial dilutions of the serum were made in 0.025 ml. volumes using HSAG as the diluent. To each dilution 0.025 ml. of antigen was added. The serum antigen mixture was allowed to react at room temperature for 1 hour. 0.025 ml. of HSAG and 0.025 ml. of 0.4% RBC suspension was added to each well, and the test was left to settle at room temperature. Included in each test were individual serum controls, RBC control, and back titration of antigen. The end-point was read as the highest dilution of serum which completely inhibited agglutination.

RESULTS

MOSQUITO INFECTION RATES

The WE infection rates in C. tarsalis are presented in table 1 and figure 1. The rates remained at or close to 100% throughout the incubation period of 31 days. Declines in the rates occurred on days 4-8 and days 14-17. The small sampling size could account for these variations.

MOSQUITO TRANSMISSION RATES

C. tarsalis transmitted WE virus 126 times out of a possible 160 (79%) attempts to mice and chicks. There were 53 out of 70 (76%) successful transmissions to mice and 73 out of 90 (81%) to chicks (table 2, and figure 2). These transmissions occurred between days 4-31 post viral infection

of the mosquitoes.

There was a gradual increase to 100% in the transmission rates to mice over the 4 week period. With regard to virus transmission to chicks there was at first a decline from 100%, and then a slow increase back to 100% within the same time period. The initial 100% transmission to chicks was probably due to a small sampling size. Initially, transmission has been found to be lower than 100% (11,38, 104,227).

C. tarsalis was found to feed more readily upon chicks than mice. This may be due to the mouse restraining cages, as mosquitoes had to probe through 2 sets of screens before they came into contact with the mice. Feeding interference was not investigated with mice but it was observed that mosquitoes more readily fed upon chicks taped to the cage bottom, leaving feet and head areas accessible, then those chicks restrained in stockings.

MOSQUITO INFECTION THRESHOLD

The WE infection threshold in C. tarsalis is presented in table 3 and figure 3. Initially, there was a slow increase in the number of mosquitoes infected with the increase in donor blood titre. After $\log_{10}$ 2.1 was reached a sharp rise occurs in the percentage of infected mosquitoes. Interpolating from the graph, a 50% infection level corresponds to a value slightly less than log 2.3 $TCID_{50}$/0.3 ml. of donor blood.

Table 1. WE infection rates in Culex tarsalis at 24°C.

Days incubation	Exp. 1.		Exp. 2.		Exp. 3.		Exp. 4.		Exp. 5.		Total	
1.	5/5[a]	100[b]					10/10	100			15/15	100
2.	1/1	100					10/10	100			11/11	100
3.	7/8	87.5					9/9	100			16/17	94
4.	9/10	90					7/7	100			16/17	94
5.	9/10	90					10/10	100			19/20	95
6.	8/8	100					9/9	100			17/17	100
7.	8/10	80					10/10	100			19/20	90
8.	10/10	100			20/20	100	10/10	100			40/40	100
9.	11/11	100	8/8	100			10/10	100			29/29	100
10.			10/10	100			8/8	100			18/18	100
11.							6/6	100			6/6	100
12.			10/10	100							10/10	100
13.					19/20	95	6/6	100			25/26	96
14.							7/7	100			7/7	100
15.							6/7	96			6/7	96
16.					18/20	90	9/9	100			27/29	93
17.							10/10	100			10/10	100

Continued .....

Table 1 Continued.

Days incubation	Exp. 1.	Exp. 2.	Exp. 3.	Exp. 4.	Exp. 5.	Total
18.						
19.						
20.			13/13 100	11/11 100	2/2 100	26/26 100
21.					3/3 100	3/3 100
22.				9/9 100		9/9 100
23.						
24.				8/8 100	6/6 100	14/14 100
25.						
26.						
27.				5/5 100	10/10 100	15/15 100
28.						
29.				9/9 100		9/9 100
30.					10/10 100	10/10 100
31.				17/17 100		17/17 100
Donor infective blood titre	5.5-6.5[c]	5.1-6.5	4.8-6.5	6.1-7.5	6.1-7.5	

[a] # of mosquitoes positive/# of mosquitoes tested.

[b] percentage.

[c] log $TCID_{50}$/0.2 ml. of blood for this one, the rest are / 0.3 ml. of blood.

Table 2. WE transmission rates by Culex tarsalis from chick[a] to chick or chick[a] to mouse.

Exp. #	CHICKS				MICE			
	Engorgement rates		Transmission rates		Engorgement rates		Transmission rates	
Days incubation								
4.	4/8[b]	50[c]	4/4[d]	100[e]	4/20	20	2/4	50
6.	15/29	52	11/15	73	14/39	36	8/14	57
8.	9/10	90	5/9	56	7/20	35	2/7	29
9.	4/10	40	4/4	100	3/10	30	3/3	100
10.	9/20	45	8/9	89	6/30	20	5/6	83
13.	15/29	52	12/15	80	9/30	30	7/9	78
16.	4/18	22	2/4	50	6/15	53	6/6	100
17.	7/10	70	6/7	86	3/8	38	3/3	100
20.	9/32	28	9/9	100	9/15	60	8/9	89
21.					3/10	30	3/3	100
24.	7/19	37	5/7	71	2/17	12	2/2	100
27.	3/20	15	2/2	100	3/17	18	3/3	100
30.	3/10	30	3/3	100	1/5	20	1/1	100
31.	2/8	25	2/2	100	0/8	0		
Total	91/223	41	73/90	81	70/244	29	53/70	76

[a] Donor blood titres ranged between log 4.8-8.5 $TCID_{50}$/0.3 ml.

[b] # of engorged mosquitoes/# of attempted transmissions.

[c] Percentage.

[d] # of hosts positive/# of positive mosquitoes.

[e] Percentage.

Table 3. WE infection threshold in Culex tarsalis.

$TCID_{50}$ of donor blood (/0.3 ml. of blood)	# of infected mosquitoes/# of engorged mosquitoes	%
Log. 0	-	-
Log. 1.1	1/5	20
Log. 1.5	2/6	33
Log. 2.1	3/8	38
Log. 2.5	13/18	72

Figure 1. WE infection rates in Culex tarsalis at 24°C.

Figure 2. WE transmission rates by Culex tarsalis at 24°C.

Figure 3. WE infection threshold in Culex tarsalis at 24°C.

Fig. 1.

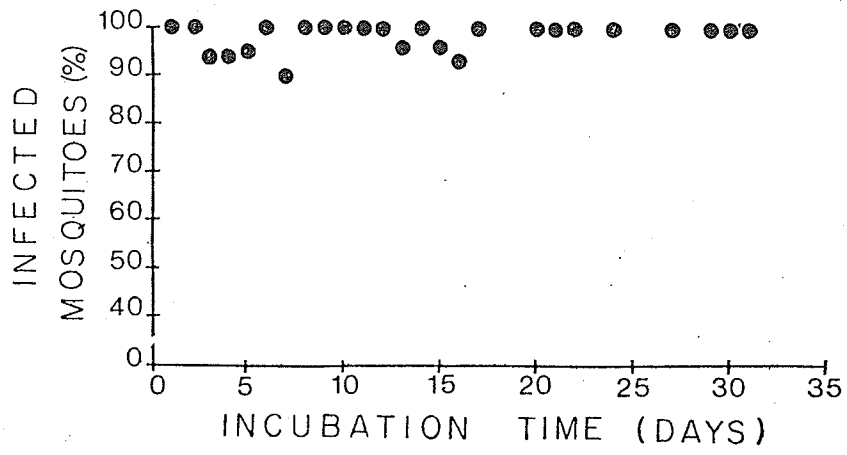


Fig. 2.

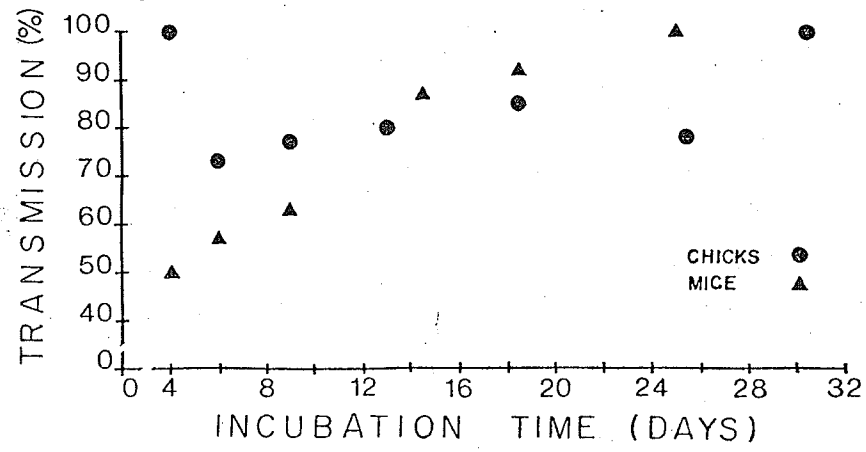
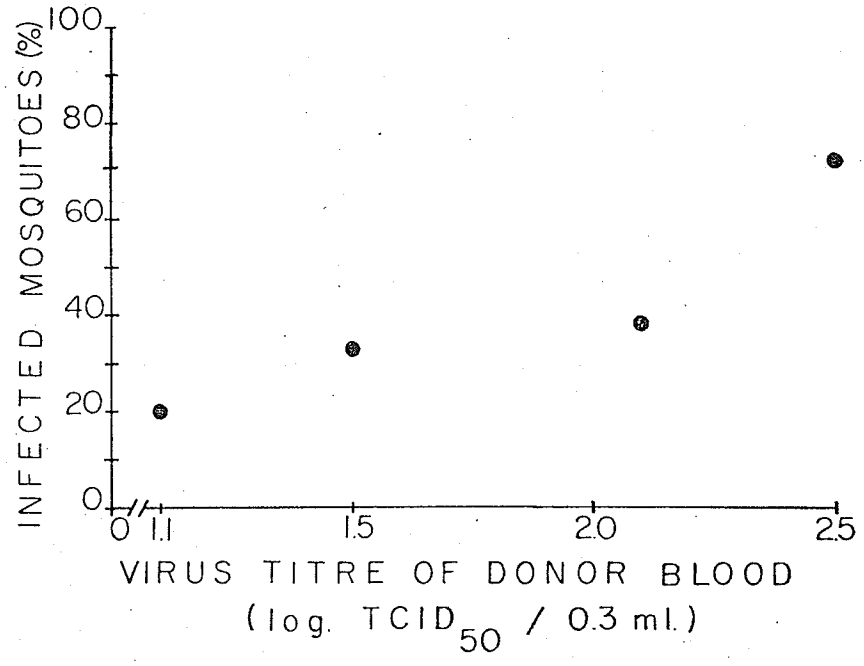


Fig. 3.



DISCUSSION

It is obvious that the Manitoba strain of C. tarsalis is readily infected with WE virus. Infection rates are maintained at or near 100%. This corresponds closely to the results obtained by Chamberlain and Sudia (38), and Hayles (104). Declines in the infection rates within the first week may be due to virus inactivation before it had infected the mosquito and begun to multiply (34,40).

Chamberlain and Sudia (40) report that mosquito infection rates of 100% are common in the laboratory. Such rates can be expected in almost any susceptible vector species provided the virus meal is well above the threshold level, and incubation is sufficient for virus growth. A high infection rate alone is not proof of vector efficiency because the presence of virus in the mosquito does not guarantee that sufficient quantities to cause disease will be inoculated during feeding (153). Infection rates are generally higher than transmission rates (183).

Mosquitoes remained infected with WE virus throughout the length of the study, which lasted 31 days. This agrees with data obtained by other researchers who concluded that C. tarsalis, once infected, remains so for life (11,16,40, 105, 185). Such long-lived WE infections can be explained by continued progressive virus multiplication in various organs together with a slow virus mortality (40).

C. tarsalis transmitted WE virus in nearly equal numbers to both chicks and mice. This mosquito species

exhibited a 81% transmission rate to chicks compared to a 76% transmission rate to mice. Hayles (104) obtained a 86% transmission rate to chicks at 24°C; very close to that found in this study. Other studies also indicate that C. tarsalis has a very high virus transmitting efficiency (38, 185).

Generally, the transmission rates to both hosts gradually increased to 100% as the incubation period lengthened. This gradual increase in transmission rates agrees with previous studies (11,38,104,227). Although it takes only a few days after virus ingestion for virus concentrations in mosquitoes to become high, it takes 2 to 3 times that incubation period for transmissions to become efficient. This is due to relocalization of the virus during incubation as well as an increase in the number of mosquitoes serving as vectors. The concentration in the salivary glands increases with a decrease elsewhere in the body of the mosquito (40) and the number of mosquitoes with infected salivary glands increases with time following infection (227).

An initial high transmission rate, such as that which occurred on day 4 in the chick experiments may be due to mechanical transmission. Mechanical transmission causes a high transmission rate initially, then a gradual decline occurs as the virus dies off, until biological transmission takes over (40). However, Hayles (104) states that biological transmissions commence after 3 days incubation. Since transmissions to mice are lower, it is most likely

that the high transmission rate (100%) to chicks on day 4 is a chance occurrence resulting from the small sample size.

The transmission experiments did not commence until the fourth day of incubation because this time period had been cited as the minimum extrinsic incubation period for WE virus in C. tarsalis (11,104,227). Virus had not been detected in the salivary glands until the fourth day (227). Also more mosquitoes were likely to feed at this time than before, and the chance of confusing mechanical transmission with biological transmission was reduced.

The 50% infection threshold, a value of log 2.3 $TCID_{50}$/0.3 ml. of donor blood obtained in this experiment corresponds closely to that found by Hayles (104) and Thomas (227), who obtained threshold values of log 2.5 LD_{50}. Barnett (11) and Hardy (95) attained infective threshold values of log 3.0 LD_{50} and 3.2 LD_{50} respectively. The infective threshold values were obtained by measuring the viremia level at which mosquitoes would transmit the virus. The infection threshold merely measures the percentage of mosquitoes infected with virus, rather than their ability to transmit it. Higher values for the infective thresholds than for the infection thresholds would be expected if the mosquito species had a salivary gland barrier, as is the case with C. tarsalis (227). Mosquitoes feeding upon donor chicks with low viremias, may not have infected salivary glands, although they may be infected with virus.

The infection threshold found in this study is low when compared with other species for the same virus (37). Mosquitoes with lower infection thresholds are better vectors because there are more hosts in nature with adequate viremia to infect them. Not only does the infection threshold vary with different species for the same virus, but field and laboratory populations of the same species vary in their susceptibility to virus infection (99). Differences were also noted between individuals within the same population. The difference in infection thresholds between the Manitoba strain and other strains of C. tarsalis is probably not significant, but even if it were, it would not be surprising.

CONCLUSION

The results of these experiments indicate that the Manitoba strain of C. tarsalis is an excellent vector of a Manitoba WE virus isolate. This mosquito species displays a high degree of susceptibility with a low infection threshold and a high infection rate. Since the extrinsic incubation period is short (4 days), many transmissions are possible. This is especially true since the virus is maintained in the mosquito's body for life. It is important that the length of the extrinsic incubation period coincides with the interval between blood-meals. This obviously indicates optimum adaption by the parasite to its vector. Transmission rates are high, indicating high infectivity of its bite. This frequency with which the bite from a single

mosquito transmits the virus is further evidence of the efficiency of C. tarsalis as a WE vector.

The fact that C. tarsalis is an efficient vector of WE in the laboratory, does not mean it is the principal vector in nature. Very little field work has been done on C. tarsalis with regards to the epidemiology of WE in Manitoba. WE has only been isolated definitely from C. tarsalis once in Manitoba (148); a total no greater than the number of isolations from C. restuans (168). Since the WE virus has also been isolated from a group of Culex species (143), it strongly suggests that this genus at least is important in WE virus propagation. Evidence from other parts of the prairies (78,153,154,204,210) further implicates C. tarsalis as being the primary vector of WE. More field surveys on the mosquito species of Manitoba, focusing on virus isolations, are needed before a definite conclusion can be made on the role of C. tarsalis in the maintenance of WE virus.

CHAPTER IV

THE BEHAVIOR OF WESTERN ENCEPHALOMYELITIS IN RICHARDSON'S GROUND SQUIRRELS

INTRODUCTION

Richardson's ground squirrels, Spermophilus richardsonii (Sabine) (RGS) have been implicated in the yearly maintenance of WE virus. The squirrels may serve as overwintering hosts (139,155) or as a spring amplifying system for the virus (137). There is adequate opportunity for contact between the RGS and the principal WE vector, C. tarsalis, as this mosquito is often found resting in mammalian burrows during the summer (100,154). However, Shemanchuk (202) did not find any mosquitoes in RGS burrows during his study.

Serological studies in Saskatchewan reveal a continuous pattern of WE infection in the RGS (137). There have also been 6 WE virus isolations from the squirrels in that province during an 11 year span (30,137). Such studies have not been conducted in Manitoba so nothing is known about the importance of RGS to WE virus maintenance in this province.

Not only the presence of WE virus in RGS is important, but also its mode of replication. Previous studies indicate that the RGS is susceptible to infection with WE virus, however symptoms and mortality rates vary depending upon age of the squirrel and the route of inoculation (75,137,220).

In the present study, I conducted a preliminary field survey of RGS in an area of the city of Winnipeg where sentinel flock seroconversion to WE had been high during 1975 (242). A study on WE replication in the RGS at 2 temperatures was also conducted.

MATERIALS AND METHODS

VIRUS

The WE virus used in this experiment was the same as reported previously (Ch. 2). Squirrels were inoculated with a log 2 dilution of virus equivalent to 1000 x $TCID_{50}$.

CELL CULTURE

Detection and titration of the WE virus was made in tubes of Vero cells. The growth and maintenance of this cell line have been previously described (Ch. 2).

RICHARDSON'S GROUND SQUIRRELS

All RGS used in the experiments were trapped in the late summer of 1976, at a farm site within the city limits of Winnipeg. They were tested for WE antibodies prior to use in experiments. During the following spring (1977), 42 RGS were trapped and sera obtained from 41 of them. Sera from both years were tested for WE antibodies by the HAI method (136). Also, in 1977, 20 blood samples, added to Alsevers, were tested for the presence of WE virus.

Three experiments were conducted with some of the squirrels caught during the fall:

1) A preliminary temperature trial at 24°C with 3 squirrels, each obtaining 0.03 ml. of WE virus. They were bled once every 4 days by cardiac puncture.

2) A preliminary temperature trial at 10°C in which 4 squirrels, injected with 0.2 ml. of virus each, were bled once every 5 days by cardiac puncture.

In both of the above experiments, the RGS (each

weighing about 300 gm.) were anaesthetized with sodium pentobarbital before they were bled. The blood samples were added to Alsevers and frozen at -70°C until assayed for virus. Preliminary trials ran for 10 days and any squirrels which died were frozen. Their brains were later removed for virus detection when time permitted.

3) An intensive study on virus maintenance was conducted using 6 squirrels kept at 24°C, and 10 squirrels kept at 10° C. Each squirrel was weighed when first bled, weights ranging between 196-536 gm. Each squirrel received 0.2 ml. of 1000 x $TCID_{50}$ dilution of virus. At the time of virus inoculation, 3 RGS were hibernating, but by the first day of bleeding all 3 had come out of hibernation. However, another squirrel had gone into hibernation by day 1 and remained so for a week. Bleeding this squirrel began on day 8. Each squirrel was bled from the retro-orbital venous plexus daily for 10 days or until death. Squirrels were anaesthetized with CO_2. When possible, 0.5 ml. of blood was added to 2.0 ml. of Alsevers and frozen. Squirrels which died were either frozen for future organ removal or operated on immediately. The following organs were generally removed for virus assay and/or slide preparation: brain, heart, lung, liver, spleen, bone marrow, brown fat, lymph node and kidney. Tissues from some of the squirrels were divided in half, 1/2 frozen for virus assay and the other half placed in formalin for slide preparation to detect viral lesions.

Any squirrels which survived the experiments were tested for WE antibodies by the HAI technique at least 1

month after WE inoculation.

VIRUS ASSAY

All blood samples and tissues were assayed on Vero cell tissue culture. Before the assay, blood samples were centrifuged for 20 minutes at 4000 rpm to remove any bacterial contamination. Tissues were assayed separately and treated before inoculation onto tissue culture as follows: Tissues were ground in 20 ml. of BHI which contained 2 x antibiotics and 10% fungizone. The mixture was transferred to a 50 ml. centrifuge tube, refrigerated for at least 1 hour, and then centrifuged at 4000 rpm for 20 minutes. The supernatant was removed and 10% FCS was added. Two ml. of this supernatant was inoculated per culture tube, and the rest frozen at -70°C. All blood samples and tissues found positive were titred by making 10-fold dilutions on Vero cell tubes.

After all inocula had been on tissue culture for 1 hour, the monolayers were rinsed with Hanks and maintenance medium was added. The tubes were observed for 6 days for signs of CPE. During titrations, $TCID_{50}$ endpoints were calculated by the Kärber method (136). The identity of isolated virus was confirmed by neutralization tests on Vero cell culture tubes. Any tissues toxic to the cell monolayer were intracerebrally inoculated into 3-week-old mice. These mice were kept for 10 days. Virus re-isolation was attempted from the brains of any dead mice. The survivors were exsanguinated and HAI test performed on the blood samples to detect WE antibody.

HISTOPATHOLOGY

Slide preparation was carried out by technicians at the Agriculture Services Complex, University of Manitoba. The technique was as follows: Tissues were fixed in formal saline for 6-24 hours depending upon size. They were then trimmed and placed in capsules which were placed in an Ultra-autotechnicon for 16 hours (table 1). Tissues in paraffin were blocked and hardened, then sectioned at 6 micron thicknesses. The sections were placed on a water and gelatin solution, then onto slides. After draining, the slides were put on a slide warmer at 16°C to ensure tissue adhesion. The slides were put through a Histo-tek slide stainer (table 2), and then covered with slips. The slides were examined for lesions by two pathologists.

Table 1. The sequence of tissue preparation in the Ultra-autotechnicon.

1.	10% formalin	5 1/2 hours
2.	10% formalin / 95% Alcohol	1
3.	95% alcohol	1
4.	100% alcohol	1
5.	100% alcohol	1
6.	100% alcohol	1
7.	100% alcohol / Xylene	1
8.	Xylene	1/2
9.	Xylene	1/2
10.	Xylene	1
11.	Paraffin	1
12.	Paraffin	2

Table 2. The sequence of events in a Histo-tek slide stainer. Each section is successively exposed to:

1. Xylene to remove paraffin from the tissue.
2. Denatured ethyl alcohol solutions to hydrate the specimen.
3. Hematoxylin to stain the nucleus.
4. Dilute hydrochloric acid to remove excess stain.
5. Denatured ethyl alcohol solution for dehydration.
6. Dilute ammonium hydroxide to enhance nuclear staining.
7. Eosin to stain cytoplasm.
8. Absolute ethyl alcohol for dehydration.
9. Xylene to clear and resin to coat the section.

RESULTS

FIELD STUDIES (Table 3)

Fifty-two RGS were caught during the late summer of 1976. All these squirrels were negative for WE antibody. Out of 20 squirrels caught the following April, none contained virus in their blood, and 1 of 19 sera tested for antibody was positive, with a titre of 1:32. The 20 squirrels were caught before major mosquito activity was reported. The size and weight of the squirrel with antibodies indicated that it was a juvenile of the previous summer, and that therefore the infection had occurred within the last year. Two additional WE antibody titres were detected in RGS caught in 1977: 1 in May and the other in July. The squirrel with WE antibodies caught in July was a juvenile of that year, and this animal yielded the highest antibody titre, a rate of 1:128.

PRELIMINARY EXPERIMENTS

24°C trial - Virus was detected in the blood samples of 3 squirrels on days 4, 6, 7 and 8. Although a detailed titration was not made, results indicated a peak in viremia on day 7, with a rapid decrease by day 8. No virus was isolated from the brains of 2 squirrels that died due to experimental mishaps. Lesions were present in the brain of the second dead squirrel. The surviving squirrel displayed no symptoms of WE infection, but it had antibodies to WE 1 month later, with a serum neutralization titre less than log 2.5 $TCID_{50}$, a drop of log 2 from the control serum. Antibodies were still present 4 months later when the HAI test revealed a titre of 1:32.

10°C trial - Virus was detected in the blood samples of 4 squirrels on days 2, 3, 4 and 9. Viremic levels were low, not exceeding log 1 $TCID_{50}$. No virus was isolated from the brains of the 2 squirrels that died due to experimental error, although lesions were present in 1 brain. Both squirrels surviving the experiment had high levels of antibodies 3 months later; 1:128 titre.

INTENSIVE STUDY

VIREMIA

There was no significant difference in the onset of viremia between the squirrels kept at 10°C and those kept at 24°C (table 4). At 24°C viremia usually started on day 1, although 1 squirrel did not experience viremia until day 6 post inoculation, and another did not exhibit any at

Table 3. The detection of HAI antibodies to WE virus in RGS captured in Winnipeg, Manitoba, 1976-1977.

Month	# tested[a]	# positive	%	titre
Sept. 76	52	0		
April 77	19	1	5.3	1:32
May 77	11[b]	1	9.1	1:16
June 77	6[c]	0		
July 77	5[d]	1	20	1:128

[a] Blood samples collected by cardiac puncture.

[b] 1 squirrel was positive to SLE virus with a titre of 1:20.

[c] 3 of the squirrels were juveniles.

[d] All of the squirrels were juveniles.

Table 4. Level of WE viremia in 0.3 ml. of blood[a] following subcutaneous inoculation[b] of the RGS.

RGS #	Days post inoculation										
	1	2	3	4	5	6	7	8	9	10	
24°C trial											
1.	1.5	-	≤.5	-	-	-	-	-	-	-	Dead on day 13.
2.	-	-	-	-	-(K)[c]						
3.	-	-	-(K)								
4.	-	-	-	-	-	-	-	-	-	-	
5.	≤.5(K)										
6.	-	-	-	-	-	≤.5(D)[d]					
10°C trial											
7.	-	-	-	-	-	-	-	-	≤.5	-	Dead 3 mos. later.
8.	-	1.1	1.5	-	-	-	-	-	≤.5	-	
9.	-	1.5	0.8	-	-	-	-	-	≤.5(D)		
10.	-	≤.5	1.1	-	-	-	-	-(D)			
11.	-	1.5	2.5	≤.5	-	-	-	-(D)			
12.	2.5	1.5	-	-	-(K)						
13.	-(K)										
15.	-	2.1	-	-	-	(K)					
16.	-	-	-(K)								

Hibernation trial	8	9	10	11	12	13	14
14.	-	-	-	-	-	-	(D)

[a] Blood samples were taken from the retro-orbital venous plexus.

[b] Each squirrel received 0.2 ml. of 1000x$TCID_{50}$ of virus.

[c] Squirrel was accidently killed on this day.

[d] Squirrel was found dead or near death on this day.

all. At 10°C, viremia generally commenced on day 2, although 1 squirrel experienced viremia on day 1, and 1 on day 9. The duration of viremia for 24°C was 1 day, while for 10°C, normally 2 days. Lowering the ambient temperature seemed to extend viremia slightly. In both groups, squirrels experienced recurring viremias. At 24°C, 1 squirrel experienced a second viremia 2 days after completion of its initial viremia. At 10°C a 6 day span occurred between viremias in 2 squirrels. Viremic titres were low in both groups, although viremias at 10°C were slightly higher than those at 24°C.

CLINICAL SYMPTOMS

Symptoms were similar at both temperatures although 1 squirrel at 24°C exhibited no signs of WE infection before death (Table 5). Virus re-isolation from its brain confirmed WE infection. Symptoms in both groups commenced on days 6 to 9 post inoculation. Symptoms consisted of tremors, involuntary salivation and, in some cases, paralysis. Of the 3 squirrels that survived the experiment, 2 did not show any signs of WE infection.

MORTALITY

Deaths occurred generally within 3 days of each other in both groups, approximately 1 week after infection, although 1 squirrel at 24°C did not die until almost 2 weeks after inoculation (Table 5). This squirrel was observed to have trouble eating, which may have caused its

Table 5. The appearance of disease symptoms in the RGS following WE infection[a].

RGS #	day of 1st symptoms	day of death	description of symptoms
24°C trial			
1.	7	13	Tremors, involuntary salivation
6.	-	6	Died without developing symptoms
10°C trial			
8.	8	survived	Tremors, involuntary salivation
9.	6	9	Involuntary salivation, paralysis
10.	7	8	Involuntary salivation, paralysis
11.	6	8	Tremors, involuntary salivation
Hibernation trial			
14.	13(6)[b]	14(7)	Involuntary salivation, paralysis

[a] Each squirrel received 0.2 ml. of 1000x$TCID_{50}$ of virus, subcutaneously.

[b] Numbers in parenthesis are the no. of days after the animal awoke from hibernation.

death. No virus was isolated from its brain.

IMMUNE RESPONSE

Few comparisons can be made between the 2 temperature groups, as only 3 squirrels, 1 at 24°C and 2 at 10°C survived the treatment. One of the 3 squirrels died, leaving only 1 squirrel at each temperature. Both squirrels had developed antibody titres, the one held at 24°C had a titre slightly lower than the one held at 10°C (1:32 compared to a 1:64). The difference between these two readings was not significant.

EFFECT OF HIBERNATION

Only 1 squirrel (#14, all tables) went into hibernation and remained so for 1 week after inoculation with WE. The squirrel died 7 days after emerging from hibernation (14 days after inoculation of WE). No viremia was detected, and the squirrel developed clinical symptoms the day before its death. The appearance of WE symptoms in this squirrel and its death corresponds to the results of the squirrels held at 24°C and 10°C.

VIRUS ASSAY OF TISSUES

Virus was isolated from brains of squirrels at both temperature conditions, as well as from the squirrel that hibernated, and from the liver of 1 squirrel (Table 6). Virus was present in the brain for 3 days, from day 5 to day 8 post inoculation. Virus titres ranged from log 2.8 to

Table 6. The recovery of WE virus from and the presence of lesions in RGS following subcutaneous inoculation[a].

RGS #	Day	Brain	Lung	Liver	Brown fat	Kidney	Heart	Spleen	Lymph node	Bone marrow
5.	1	-	-	-	ND[e]	-	-	-	ND	ND
13.	1	-	-	-	ND	-	-	-	ND	ND
3.	3	-	-	-	ND	-	-	-	ND	ND
16.	3	-	-	+	ND	-	-	-	ND	ND
2.	5	3.8/+[b]	-/+	-/NVL	-/+	-/CI[f]	-/NVL	-/CI	-/CI	ND/CI
12.	5	5.2/+	-/+	-/+	-/+	-/NVL	-/+	-/CI	-/CI	ND/CI
6.	6	4.2[c]	-	-	ND	-	-	-	ND	ND
15.	6	4.2	-	-	ND	-	-	-	ND	ND
10.	8	4.5/+	-/NVL[d]	-/NVL	-/NVL	-/NVL	-/NVL	-/+	-/NVL	ND/+
11.	8	4.5/+	-/NVL	-/NVL	-/NVL	-/NVL	-/NVL	-/NVL	-/NVL	ND/NVL
9.	9	/+	-/+	-/+	ND/+	-/+	-/NVL	-/NVL	ND	ND/NVL
1.	13	-	-	-	ND	-	-	-	ND	ND
14.	14	2.8	-	-	ND	-	-	-	ND	ND

[a] Each squirrel received 0.2 ml. of 1000x$TCID_{50}$

[b] Virus/lesion

[c] Virus titre/0.3 ml. of brain

[d] NVL - no visible lesion

[e] ND - not done

[f] CI - can not interpret

log 5.2 $TCID_{50}$/0.3 ml. of brain tissue (Table 6). Of the 10 tissues assayed in 3-week old mice, 1 yielded the liver isolate. The corresponding squirrel (16) was held at 10°C. Titration was not attempted due to the toxicity of this tissue specimen.

HISTOPATHOLOGY

Both temperature groups are considered together due to the small sample size; only 1 squirrel from the 24°C group was examined for virus effects. Lesions were found in the following tissues: brain, lung, liver and brown fat (Table 6). Questionable lesions were detected in the spleen, kidney, heart and bone marrow. Lesions were consistently found in the brain; 4 of 5 yielding virus. No virus was isolated from any of the other tissue containing lesions.

BRAIN (Figures 1-4)

Lesions were found in all areas of the brain, but particularly in the cerebral cortex. Large numbers of predominantly mononuclear cells were found in the meninges, particularly near blood vessels. Numerous foci of acute necrosis were observed throughout the brain. A mixed inflammatory reaction of granulocytes and mononuclear cells was associated with these areas. Some gliosis and neuronophagia were seen. Mild to moderate perivascular cuffing was observed near the foci of necrosis.

Figure 1. A section of normal RGS brain (x63).

Figure 2. A section of a RGS brain showing mononuclear perivascular cuffing (small arrow) and some gliosis (large arrow) (x63).

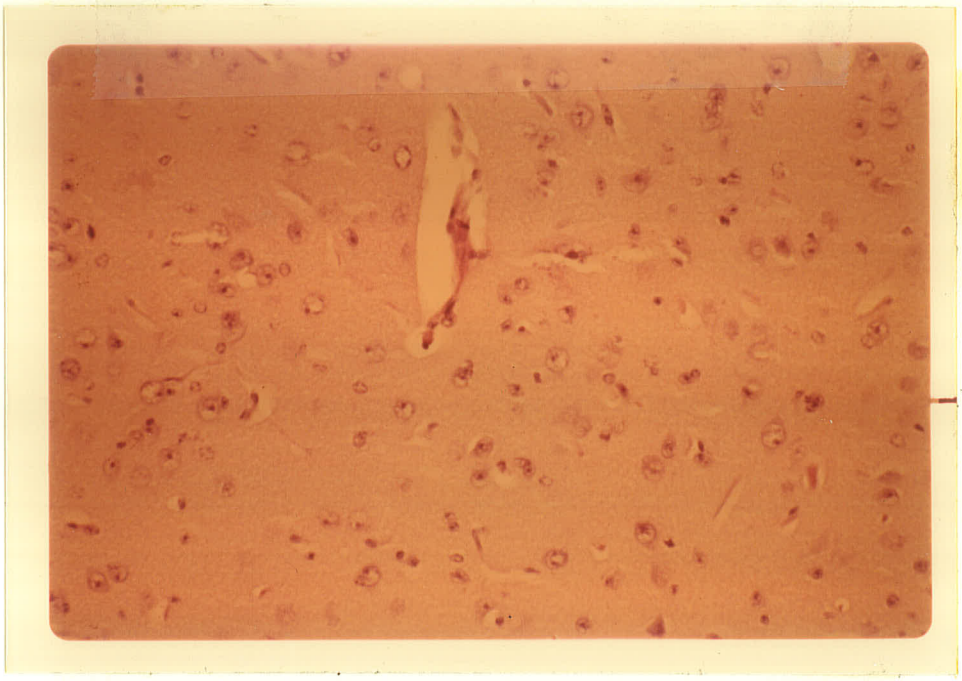


Fig. 1.

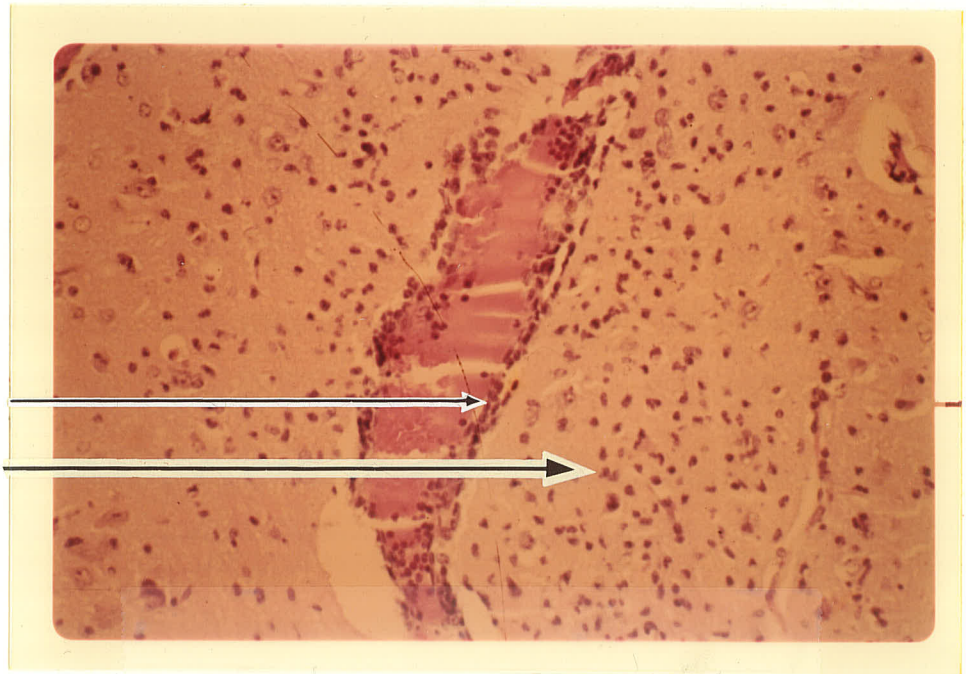


Fig. 2.

Figure 3. A section of the brain showing focal necrosis with mononuclear and granulocytic inflammatory reaction (x63).

Figure 4. A section of the meninges of the brain showing mononuclear inflammatory reaction (x63).

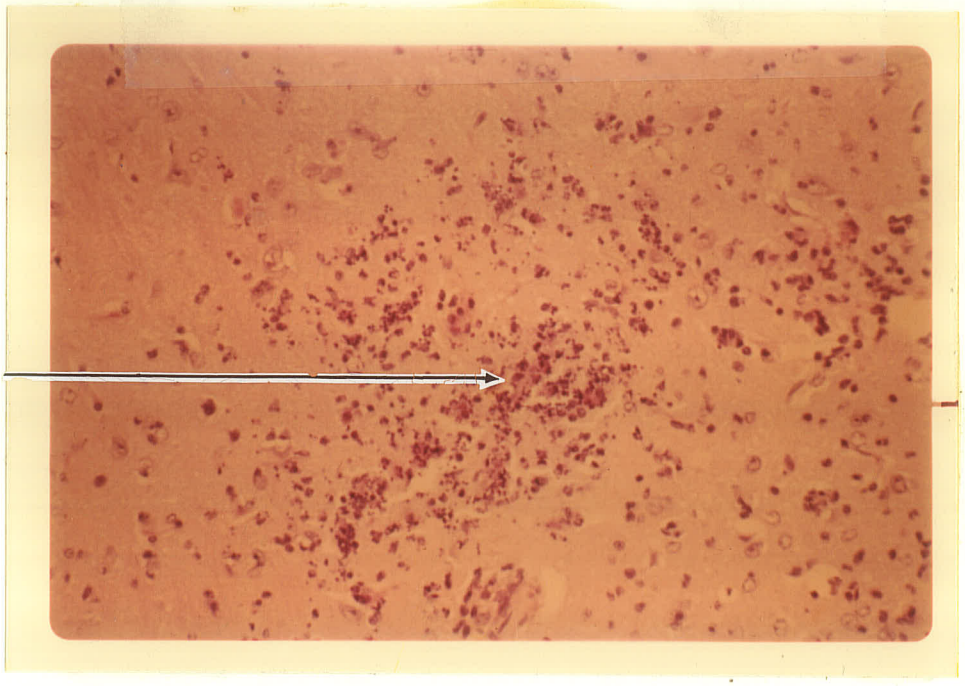


Fig. 3.

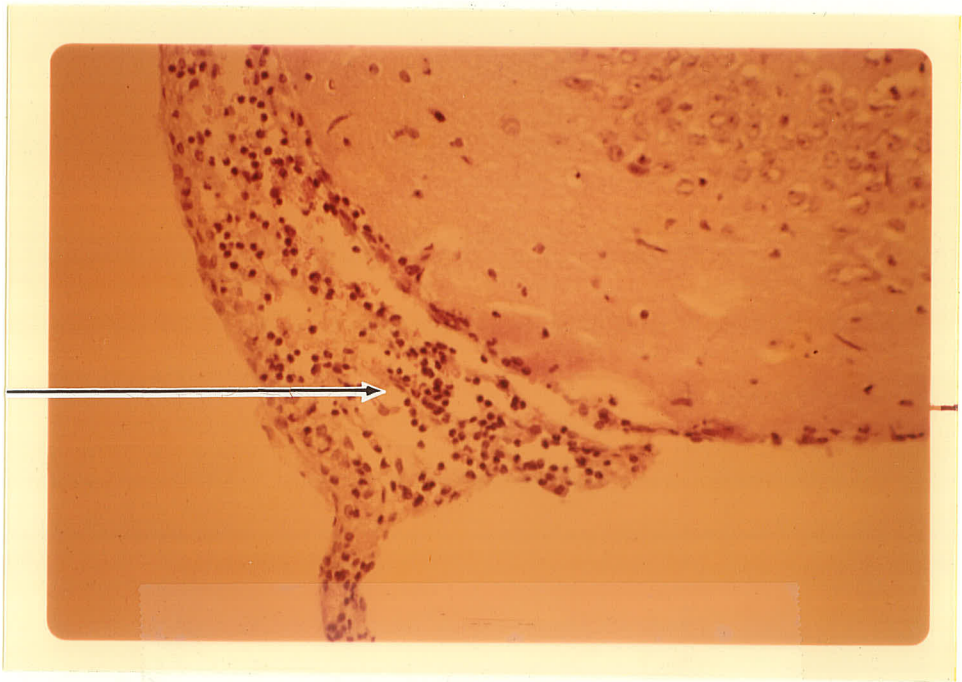


Fig. 4.

Figure 5. A section of normal lung (x63).

Figure 6. A section of lung, showing pneumonitis with macrophages (small arrow) and foci of necrosis (large arrow) (x63).

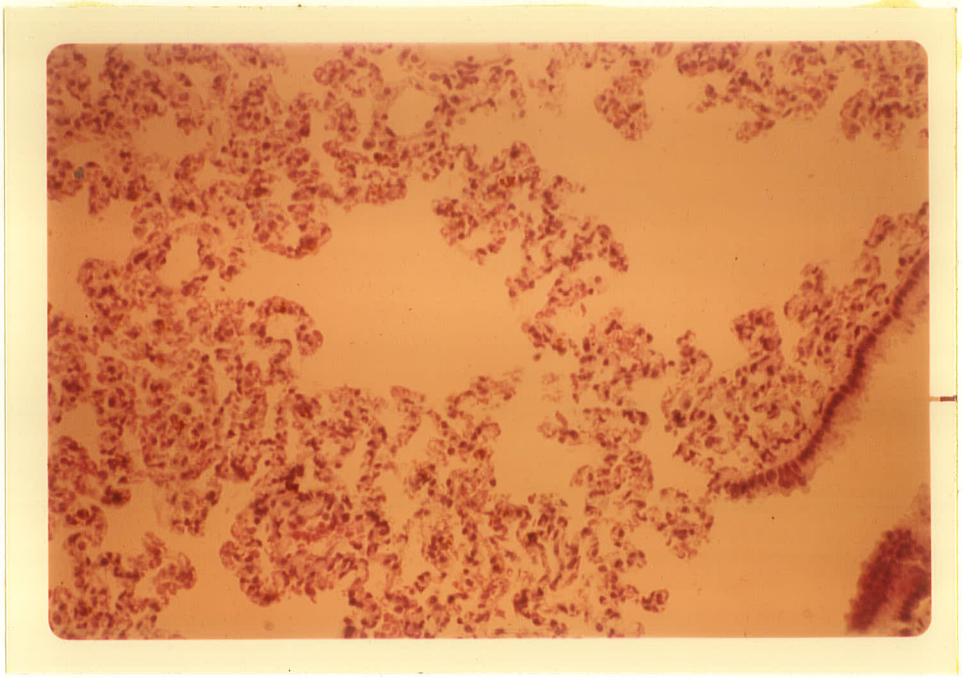


Fig. 5.

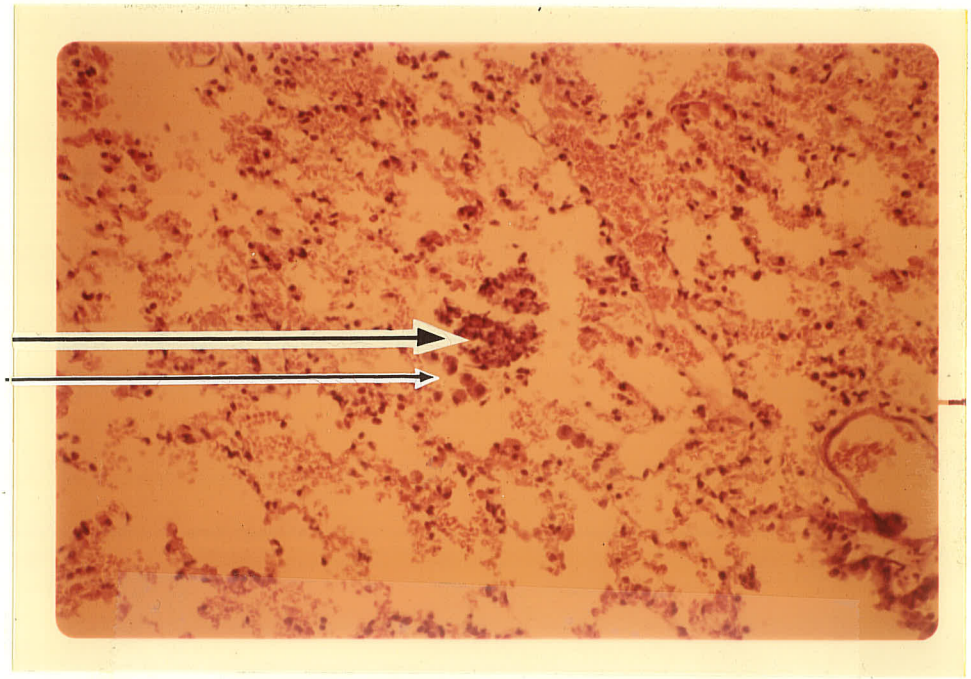


Fig. 6.

Figure 7. A section of normal liver (x63).

Figure 8. A section of liver showing focal necrosis and inflammatory reaction (x63).

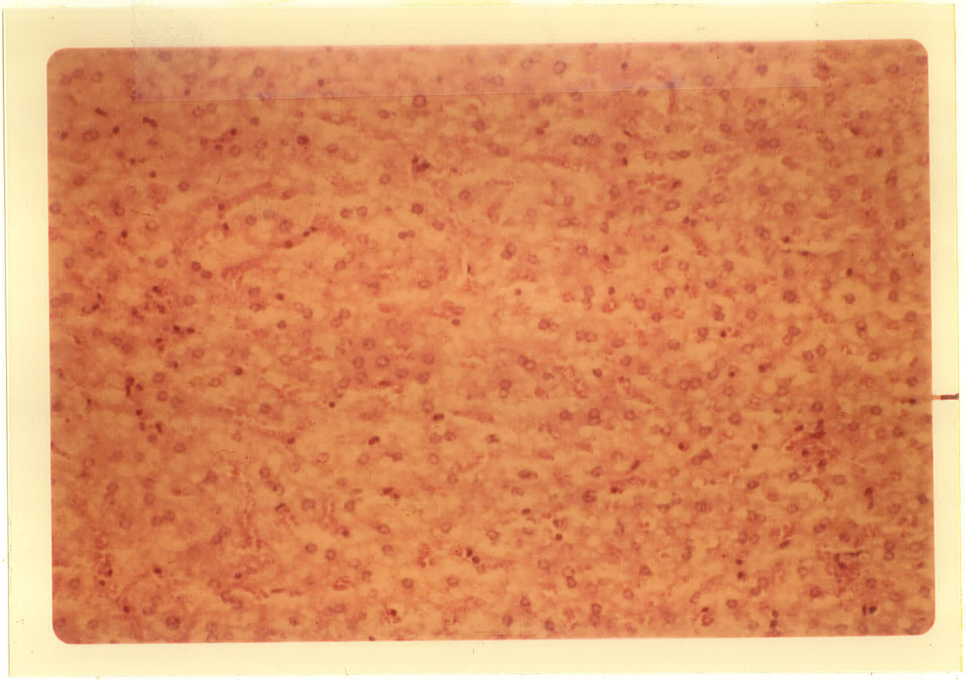


Fig. 7.

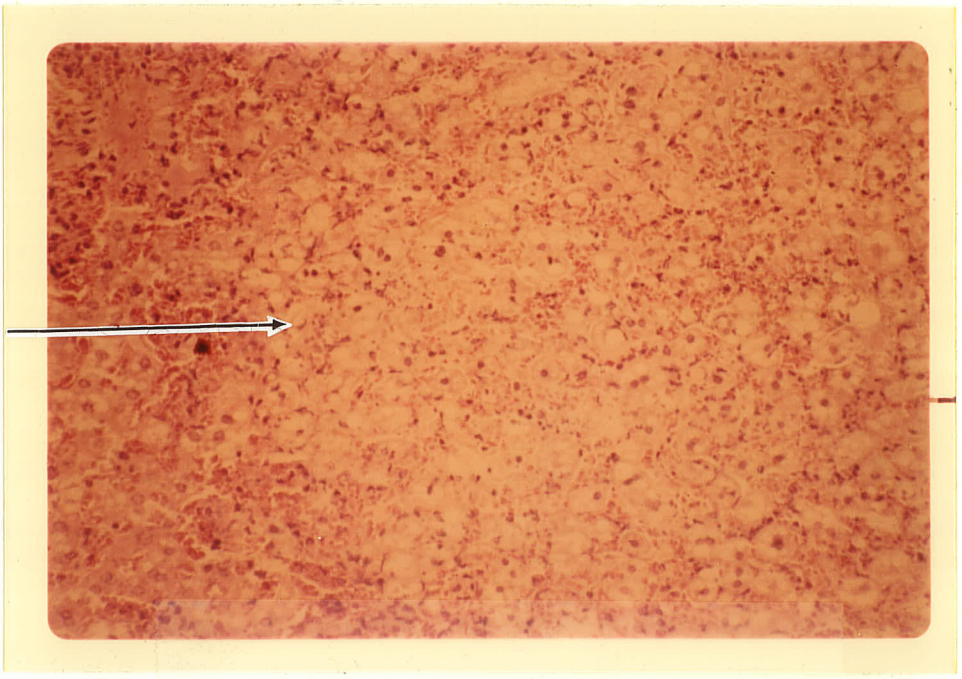


Fig. 8.

Figure 9. A section of normal brown fat (x63).

Figure 10. A section of brown fat showing focal steatitis (x63).

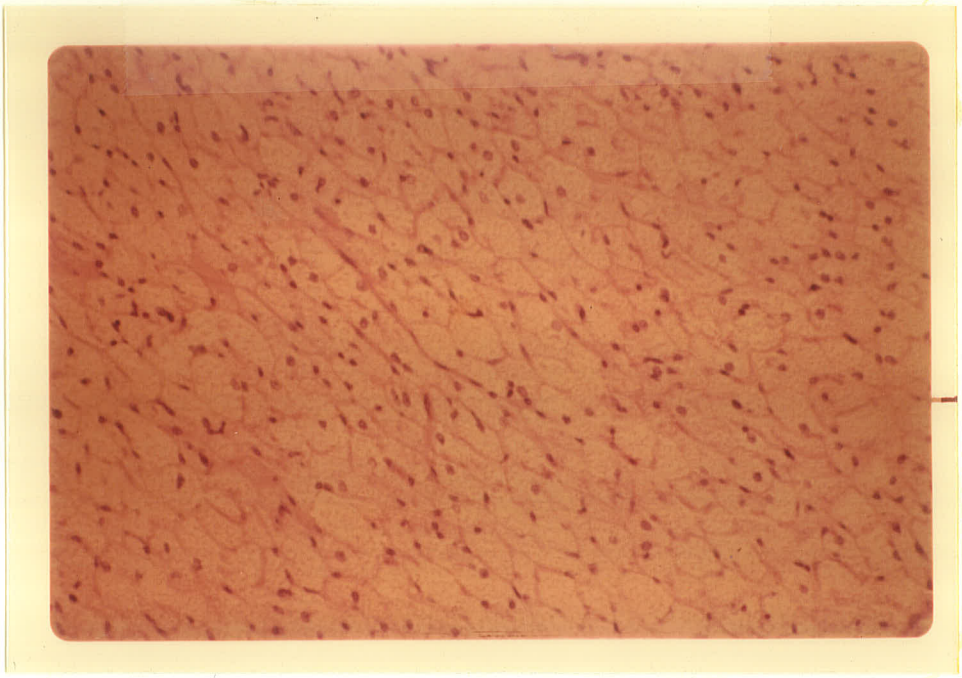


Fig. 9.

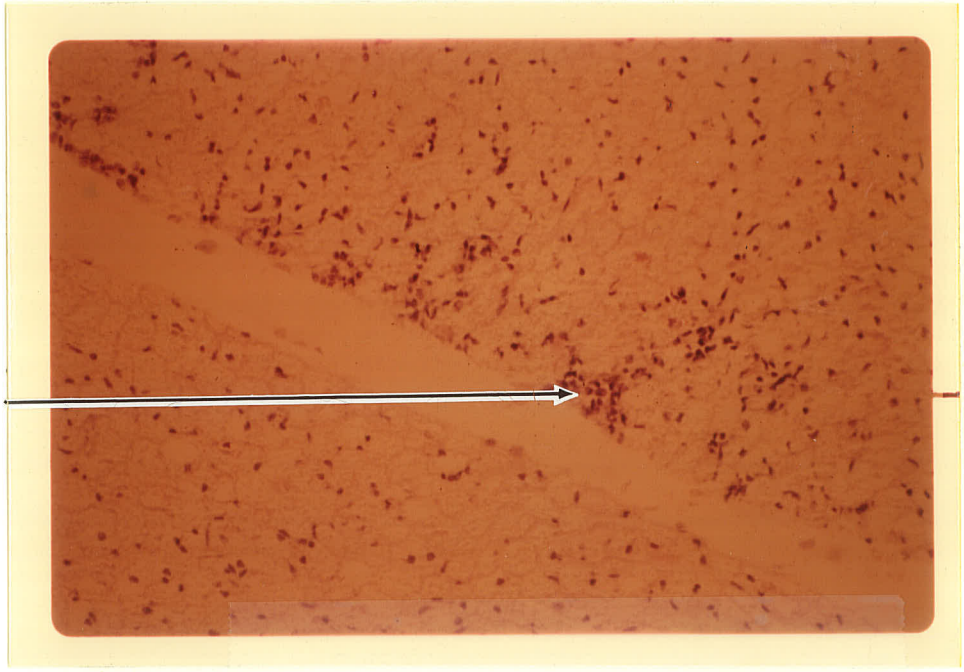


Fig. 10.

LUNG (Figures 5-6)

Neutrophils and debris were observed in moderate numbers in alveoli and some terminal branchioles. Many of the alveolar walls were thickened due to mixed inflammatory infiltration. A few foci of acute necrosis were observed throughout the lung.

LIVER (Figures 7-8)

A few foci of acute necrosis with infiltrating granulocytes were observed throughout this tissue.

BROWN FAT (Figures 9-10)

Small areas of focal necrosis with mononuclear infiltration and interstitial hemorrhage were observed in this tissue.

OTHER TISSUES

One section of bone marrow was hypercellular, and one heart section displayed mild hyperplasia of vascular endothelium. One splenic section displayed slightly activated follicles, with periphery of these follicles and the red pulp infiltrated by granulocytes. The kidney section contained mild chronic interstitial nephritis confined to the medulla.

DISCUSSION

During April 1977, 5.3% (1/19) of the RGS caught were positive for WE antibody. In May the rate was 9.1% (1/11), in June 0% (0/6) and in July 20% (1/5). During May, the first squirrel yielding SLE antibody in Manitoba was detected. The WE antibody rates for April, May and July are high, while the zero rate in June is low when compared to a previous study in Saskatchewan (137). The small sample size of my study may be the reason as the Saskatchewan study involved many more animals. The lack of antibodies in the late summer capture of the previous year (1976) corresponds to data cited by Leung (137) who obtained zero conversion rates for August.

The lack of virus isolations from the blood samples of 20 squirrels caught in April 1977 is not surprising. During a ten year study in Saskatchewan (137) only 2 WE virus isolations occurred from the blood samples of RGS. One such isolation took place during the epidemic year of 1965. Three additional virus isolations from RGS brains occurred during that study. WE virus has been isolated from brains of RGS in Saskatchewan on other occasions (76,77).

Such virus isolations and antibody titres indicate that RGS may come into contact with infected vectors of encephalomyelitis. The use of the squirrels as monitoring systems for early seasonal amplification has been suggested (139). The importance of RGS in the epidemiology of WE

virus in Manitoba cannot be determined from the field study due to the small sample size. However, 3 squirrels were positive for WE antibody and this indicates that these mammals are potential hosts of the disease in Manitoba.

Temperature, ie lowering or raising the ambient temperature of animals, can influence the course of an arbovirus infection (9,142,165). Lower temperatures may result in more virulent pathogenicity of some viruses, or if they cause reduced metabolic activity in the host, they may suppress virus activity. The high mortality rates found in the squirrels held at 10°C compared to squirrels Leung (137) held at 20°C would indicate that lowering the environmental temperature of RGS increases the virulence of the disease. However, the local isolate of WE virus was virulent for the squirrels held at 24°C as well. This may indicate a more virulent strain of WE compared to the one used by Leung (137). Since the sample size in my experiments is small, no definite conclusions can be made regarding this observation.

During a study on WE infection in the Snowshoe hare (126), fluctuating temperature and humidity affected virus replication. Hares, held at 2 different temperature ranges, experienced an accelerated viremia and an increase in duration and titre when they were exposed to fluctuating temperature and humidity. Under constant conditions, both groups showed similar viremia response. This appears to

have occurred in my experiment with RGS as well as in Leung's (137) research with RGS. No marked difference in viremia or other factors, was observed at the constant temperatures experienced by the squirrels. Before extrapolating to field conditions the environmental effects on the results must be considered.

In contrast, hibernation did have an obvious effect upon the WE virus replication. During the week after the squirrel emerged from hibernation, it developed symptoms and died in about the same length of time as the squirrels not hibernating. One would have to conclude therefore that viral growth was arrested in this animal during the hibernation period. Other research (138) has indicated the same results. The infected squirrels emerged from hibernation and developed a fulminating, fatal encephalitis, as occurred with 1 squirrel in my study.

Since no significant or consistent differences were noted between the 2 temperature groups, they will now be discussed together.

Although the preliminary trials indicated that viremia occurred for 3 to 4 days at both temperatures, this was not found in the intensive study. The longer viremias in the former trials may have been due to sampling a different squirrel each day. Viremia in the latter study was of short duration (1-2 days) for both 24°C and 10°C. Titres were low. Since viremic duration was short, peaks of viremia were difficult to interpret. One squirrel, that

experienced viremia for 3 days, had a peak titre on the second day. Other research on RGS (137) and the California ground squirrel (97) indicated longer viremias of 5 days duration with peak titres on day 2 or on days 2-4 respectively. Intramuscular inoculation of WE virus into 3-week-old mice usually resulted in viremias of 3 days duration (66).

Subcutaneous inoculation does not always result in a detectable viremia (97,137), as occurred in this experiment. Mims (162) states that there is a viremic state in virus diseases, whether detectable or not. In arbovirus infections, viremia is important as adequate viremia levels are essential for the spread of virus to new hosts. The short duration of viremias and the low virus titres obtained in my experiment indicate a poor host-parasite relationship. This has also been noted in the eastern cottontail rabbits when experimentally inoculated with eastern encephalitis (102). Only 20% of these animals developed viremias.

The indication of recurring viremias in RGS in my experiment is interesting. No other research on mammals has mentioned this. Snakes recycle virus in their blood (29,72), and birds may experience latent infections (189). Cyclic viremia increases the potential of a host as an important reservoir. Although cyclic viremia is indicated in my experiment, titres were low and, consequently, may be of no significance.

In arbovirus infections, viremia precipitates the clinical symptoms (63). Generally in this experiment, viremia occurred during the first 5 days following WE inoculation. Symptoms commenced around the end of the first week following infection, normally resulting in death a few days later. In fact, only 1 of 7 squirrels showing signs of infection survived the experiment. The symptoms were consistent with CNS disturbance (137,207), and have been found in previous experiments with RGS (137,220), mice and guinea pigs (161,197). WE infection does not always result in marked clinical symptoms (75,137,197) as was found in 3 squirrels in my experiment.

Mortality of infected RGS was high; 67% (6/9) of the animals died. (A study carried out by the Department of Zoology, University of Manitoba, indicated RGS adapted well to confinement, with low mortality rates). High mortality rates contradict Leung's work (137) in which only 27% (3/11) of her adult animals died. Mortality rates depend upon age, dosage, and route of inoculation (137,140,198). Intracerebral and intranasal inoculations are usually fatal (75,115,137,220), while subcutaneous inoculation is not (97, 137,140). In adult squirrels (137) and mice (140) mortality is not dose dependent. Therefore, although the virus dosage received by my squirrels is high compared to what they receive in nature, the squirrels can still be considered highly susceptible to WE infection. Consequently they can

only play an incidental role in the maintenance of WE virus in nature (135).

Arbovirus infections result in life-long immunity (63). All squirrels surviving my experiments developed antibodies to WE virus. With EE virus long-term antibody production following a single inoculation of live virus appears to be a species characteristic (102). High antibody titres in chickens, humans and a rabbit appeared throughout the test period, while antibodies in pigeons declined. Those in a turtle and a garter snake disappeared. This pattern of antibody development may also be true for WE virus.

One of the surviving squirrels did not exhibit detectable viremia during the experiment. The detection of antibodies, however, indicated that it had been infected. Consequently, the presence of antibodies in an animal does not prove that the species produces viremia adequate for the infection of arthropod vectors.

The distribution of virus in the tissues was not widespread. Seven brains and 1 liver yielded virus. Leung (137) found both the brain and the lymph node frequently infected with WE virus. No lymph nodes in my experiment contained detectable virus.

The first tissue found positive for virus was the liver on the third day after inoculation. This is the same time as Leung (137) initially detected virus in any of the

tissues she studied. However, her first positive tissue was a kidney.

WE virus was re-isolated from the brains of 4 squirrels prior to onset of clinical symptoms, and from 3 squirrels that died 1 to 2 days after the initial appearance of these signs. No virus was re-isolated from the brains of 2 squirrels that died 3 and 6 days after onset of symptoms. The chances of recovering virus from the brain are greater during the first or second day of clinical disease than later on (159). However, late brain isolations have been recorded in both RGS (137) and the California ground squirrel (97). WE virus was re-isolated from the former 12 days after inoculation, and from the latter 14 days after inoculation. The brain is obviously the prime target of WE infections. This is verified by the larger number of WE isolations as well as higher titres than in other tissues (66,97,137,140).

Lesions in the brain consisted primarily of perivascular cuffing, foci of acute necrosis and mixed inflammatory reaction; all characteristic of WE infection (32). Following WE infection, the brains of horses have yielded similar lesions (118,159,160,161,164). The lesions resemble those caused by the other equine encephalitis, although they may not be as intense (8,63,121,207). Leung (137) found the same principal areas of the RGS brain affected by WE infection as were found in my study:

meninges, brain stem and cerebral cortex.

Lesions due to WE infection in non-nervous tissue have not been studied to the extent that brain lesions have been studied. Adipose tissue of white mice displayed necrosis and inflammation after WE infection (2). In another study (140), the lack of appreciable inflammatory changes in non-nervous tissues was thought due to a very rapid infection process which did not allow sufficient time for much inflammation or other morphologic changes to occur. The tissues studied in the above experiment included skin and muscle collected from around the site of inoculation. In fact, pathologic changes of the CNS of these mice were minimal. This indicated the infection process in mice was much more rapid than in RGS since the latter displayed marked lesions in the brain, as well as in other tissues.

In my experiment, lesions obtained from tissues may not be due to the WE infection. These animals were not raised in disease-free environments; consequently the lesions can be due to previous infections other than WE. Only in those tissues from which virus has been actually isolated, can the lesions be assumed due to WE infection. A presumptive diagnosis can be made only upon finding such lesions in tissues. Virus re-isolation is necessary for confirmation of infection (207).

CONCLUSION

The results of this experiment indicate that RGS are not very good reservoirs of WE virus because they are highly susceptible to the disease, viremias are low and short, and inoculation does not always result in detectable viremia. Available results indicate that hibernation arrests viral replication. Further work on the interaction of hibernation and virus growth is warranted. The effect of temperature upon virus infection needs further investigation if definite conclusions are to be made. It is possible to demonstrate recurring viremias in a small number of these animals. Further research in this area is needed. Since antibodies will result from an infection in which no detectable viremia occurs, the serologic survey of wild-caught squirrels indicates only that the animals come into contact with the disease and not that they are efficient hosts of WE virus.

CHAPTER V

SUMMARY

The purpose of this research was to study the transmission of a local isolate of WE virus by a Manitoba strain of C. tarsalis, and the effect of temperature on the WE viral replication in the RGS. The results indicate that future work in some areas is still needed. The conclusions are summarized as follows:

1) C. tarsalis is unable to transmit WE virus transovarially to its offspring. Unless more sensitive methods for detecting virus are developed, the continued study of this mechanism of transmission in C. tarsalis is not advised. However, other mosquito species can be studied in this respect, especially the Aedes species that overwinter in the egg stage.

2) Manitoba C. tarsalis is an efficient laboratory vector of WE virus. To establish its importance in the epidemiology of WE virus in Manitoba, more field surveys are necessary. Such field work should include the testing of mosquito species for virus as well as their association with hosts in which the infection is found to occur. Only when C. tarsalis is compared with other species of mosquitoes can its specific importance be determined. Laboratory analysis of the vector competence of other species found infected in nature will also be needed.

3) The Richardson's ground squirrel may not be as important a host of WE virus as some researchers believe.

WE inoculation does not result in optimum viremia and high mortality rates occur. The effects of temperature on WE viral replication in the squirrels is not clear, indicating a need for more research in this area. The use of fluctuating, rather than constant temperatures in these experiments is advised. Future research on the effects of hibernation is needed, especially since RGS are considered as possible overwintering hosts. The field survey indicates that these squirrels do become infected with the disease in nature. A more intense serologic survey of RGS is advised, as these squirrels can still serve as monitoring systems for WE surveillence.

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APPENDIX

COMPOSITION OF MEDIA AND SOLUTIONS

MEDIA

	Concentration (mg/L) # 1969 (Tissue culture)	MEM (Virus assay)
L-Alanine	25	
L-Arginine	58	105
L-Aspartic acid	30	
L-Cysteine.HCl	0.10	
L-Cystine	20	24
L-Glutamic acid.H_2O	67	
L-Glutamine	200	292
Glycine	50	
L-Histidine	16.2	31
L-Hydroxyproline	10	
L-Isoleucine	20	52.50
L-Leucine	60	52.40
L-Lysine	70	58
L-Methionine	15	15
L-Phenylalanine	25	32
L-Proline	40	
L-Serine	25	
L-Thereonine	30	48
L-Tryptophan	10	10
L-Tyrosine	40	36
L-Valine	25	
Valine		46
p-Aminobenzoic acid	0.05	
Ascorbic acid	0.05	
d-Biotin	1	
Calcium pantothenate	1	
D-calcium pantothenate		1
Choline chloride	1	1
Folic Acid	1	1
Glutathione	0.05	
i-Inositol	2	2
Nicotinamide	1	1
Pyridoxal.HCl	1	1
Riboflavin		0.10
Riboflavin-5-phosphate	0.10	

Continued.....

	Concentration (mg/L) # 1969 (Tissue culture)	MEM (Virus assay)
Thiamine.HCl	1	1
D-Glucose	1000	
Glucose		1000
Phenol red	20	10
NaCl	8000	6800
KCl	400	400
$CaCl_2$	140	200
$MgSO_4.7H_2O$	200	200
Na_2HPO_4	180	
$NaH_2PO_4.H_2O$	70	140
$NaHCO_3$	560	2200

ALSEVER'S SOLUTION

Glucose	2.05 gm
Sodium citrate	0.8 gm
Citric acid	0.055 gm
Sodium chloride	0.42 gm
Distilled water	100 ml

Sterilize by autoclaving at 10 lbs/10 minutes.

TRYPSIN

Sodium Chloride	80.0 gm
Potassium Chloride	4.0 gm
Dextrose	10.0 gm
Sodium Bicarbonate	5.8 gm
Trypsin (Difco 1:250)	5.0 gm
Versene (EDTA)	2.0 gm
Phenol red (0.5%)	4.0 ml

Bring to 1 liter of distilled water and filter.

HANKS' WASHING SOLUTION

Sodium Chloride	48.0 gm
Potassium Chloride	2.4 gm
Magnesium Sulfate	1.2 gm
Calcium Chloride	0.84 gm
Sodium Phosphate	0.36 gm
Potassium Phosphate	0.36 gm
Dextrose	6.0 gm
Sodium Bicarbonate	2.1 gm
Phenol red (0.5%)	12.0 ml

Volume: 6 liters before adding last 3 ingredients.
Adjust pH to 7.0.

FORMAL SALINE

Formalin (40% formaldehyde)	2000 ml
Sodium chloride	170 gm
Distilled water	18 liters
Acid sodium phosphate (monohydrate)	80 gm
Anhydrous sodium phosphate	130 gm